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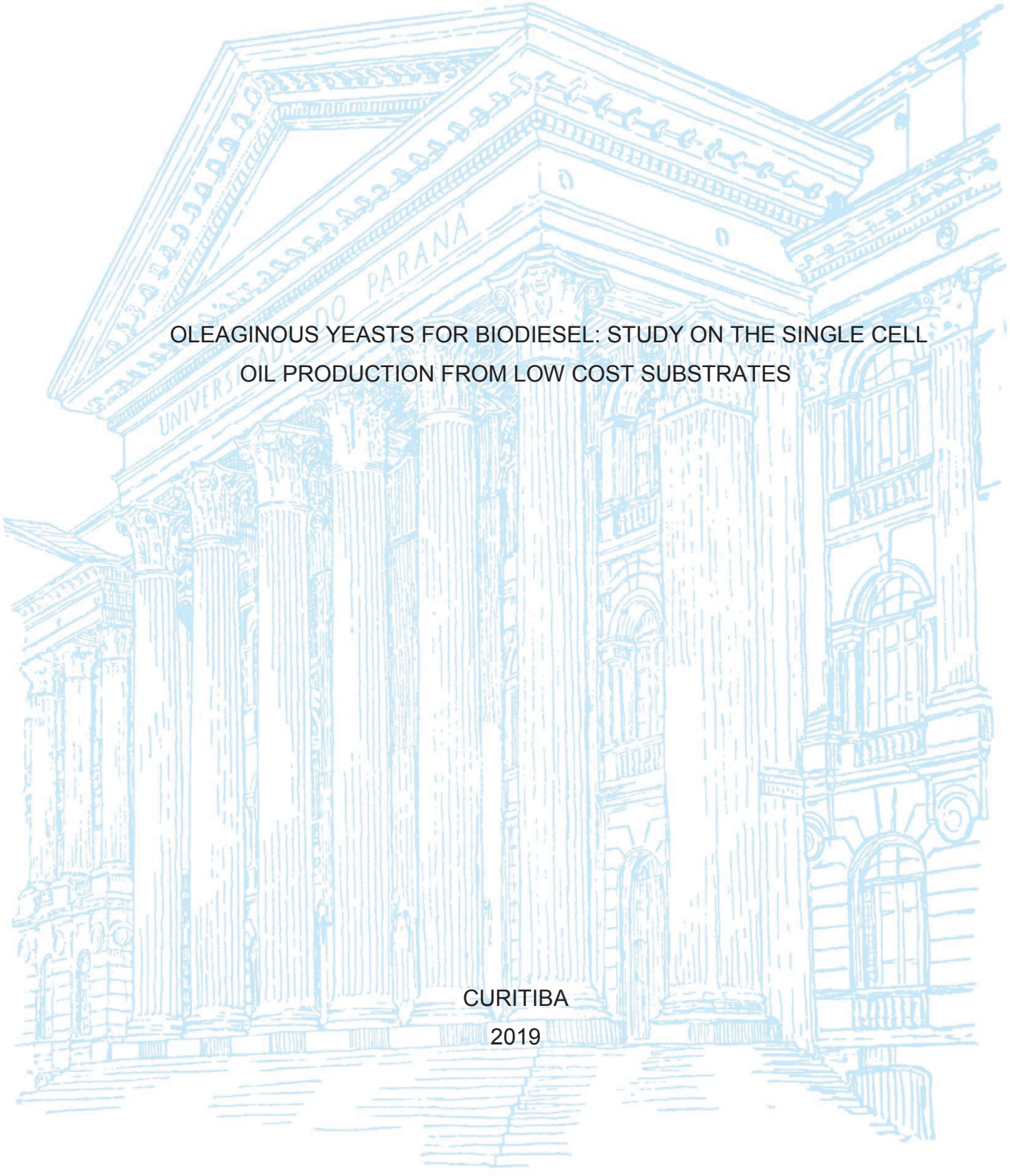

OLEAGINOUS YEASTS FOR BIODIESEL: STUDY ON THE SINGLE CELL OIL PRODUCTION FROM LOW COST SUBSTRATES

CURITIBA

2019

BIAANI BEEU MARTÍNEZ VALENCIA

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná.

Orientador: Prof. Dr. Carlos Ricardo Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **BIAANI BEEU MARTINEZ VALENCIA** intitulada: **OLEAGINOUS YEASTS FOR BIODIESEL: STUDY ON THE SINGLE CELL OIL PRODUCTION FROM LOW COST SUBSTRATES**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 27 de Outubro de 2017.

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RESUMO

O presente trabalho foi dividido em três capítulos, a primeira parte do trabalho foi uma revisão da literatura sobre o biodiesel, resíduos agroindustriais (melaço de cana e cachos vazios dos frutos secos da palma (*Elaeis guinensis*)) e sobre o potencial de microrganismos oleaginosos. No primeiro capítulo, avaliamos a capacidade de 140 cepas isoladas de leveduras por acumulação de lipídios de diferentes ambientes, coletadas no Estado do Paraná, Brasil. No screening revelou sete cepas que foram identificados como potenciais produtores de lipídios. Verificou-se que PPGEBB115 acumulou o maior teor de lipídios, até 32,06%. Entre as cepas testadas *R. glutinis*, teve a melhor produtividade lipídica, 0,456 g/L/dia. Além disso, 4 leveduras isoladas foram avaliadas em glicose, xilose, glicerol, melaço de cana de açúcar e hidrolisado dos cachos vazios dos frutos secos (OPEFB), *R. glutinis* apresentaram melhor produtividade (0,48 g/L/dia) utilizando o melaço de cana e *R.* mucilaginosa apresentaram melhor produtividade de 0,24 g/L/dia de hidrolisado de OPEFB, o teor de lipídios foi 33,04% e 17,80%, respectivamente. Os resultados mostram que as leveduras isoladas podem ser candidatos promissores para a produção de óleo. No capítulo dois, a utilização do hidrolisado de OPEFB como substrato para a produção de óleo foi estudado. O hidrolisado tem pentoses (21,19 g/L) e hexoses (0,72 g/L), juntamente com outros produtos de degradação tais como ácido acético, ácido fórmico, furfural e furfural hidroximetil (HMF). Os estudos indicaram que o hidrolisado detoxificado com carvão ativado, apresentam uma diminuição considerável de concentração dos compostos inibidores, reduzindo 91,52% de HMF, 92,63% de furfural, 33,48% de ácido acético e 14,48% de ácido fórmico. Usando o hidrolisado apresentou um crescimento e produção de lipídios de *R. mucilaginosa* com 5,11 g/L e 17,26%, respectivamente. R. mucilaginosa cresceram bem com hidrolisado destoxificado num reator por lotes de 10 L, com uma relação C/N de 19,85, obteve um rendimento de lipídios e teor de lípidos atingido 1,78 g/L e 14,62%, respectivamente. Foi estudado o cultivo de alta densidade celular com estratégia de alimentação de nitrogênio em dois estágios para melhorar a concentração lipídica e conteúdo lipídico, obtendo-se 4,85 g/L e 19,75%. E o último capítulo, o melaço de cana foi utilizado como substrato para a produção de lipídios utilizando *R. glutinis*. A adição de extrato de levedura com sulfato de amônio e Tween 20 aumentou em 27% o acúmulo de lipídios. A condição ótima utilizando a metodologia de superfície de resposta (MRS) para obter máxima concentração de lipidios foi com uma concentração de melaço de 5,83 % e 1,78 g/L de sulfato de amônio, durante 6 dias. A produção de lipídios foi melhorada num bioreator de tanque agitado com pH de 5,5, taxa de aeração de 1,5 vvm e 1,5 g/L com Tween 20. Na fermentação descontínua, foi possível obter uma produção de biomassa de 27,95 g/L, um conteúdo lipídico celular de 60,94% e 3,40 g/L/dia de produtividade lipídica.

Palavras-chave: Óleo de uma única célula (SCO). Microrganismos isolados. Biodiesel. Melaço de cana. Hidrolisado dos cachos vazios de frutos secos (OPEFB). Otimização de bioprocessos.

ABSTRACT

The present work was divided into three chapters, the first part of which was a review of the literature on biodiesel, agroindustrial residues (sugarcane molasses and empty palm nuts (*Elaeis guinensis*)) and on the potential of oleaginous microorganisms. In the first chapter, we evaluated the capacity of 140 strains isolated from yeasts by the accumulation of lipids from different environments, collected in the State of Paraná, Brazil. In the screening revealed seven strains that were identified as potential producers of lipids. It was found that PPGEBB115 accumulated the highest lipid content, up to 32.06%. Among the tested strains *R. glutinis* had the best lipid productivity, 0.456 g / L / day. In addition, 4 isolated yeasts were evaluated in glucose, xylose, glycerol, sugarcane molasses and hydrolysates of the dried fruit bunches (OPEFB), *R. glutinis* presented better productivity (0.48 g/L/ day) using sugarcane molasses and *R. mucilaginose* presented better productivity of 0.24 g / L / day of OPEFB hydrolyzate, the lipid content was 33.04% and 17.80%, respectively. The results show that isolated yeasts may be promising candidates for oil production. In chapter two, the use of the OPEFB hydrolyzate as a substrate for oil production was studied. The hydrolyzate has pentoses (21.19 g / L) and hexoses (0.72 g / L) along with other degradation products such as acetic acid, formic acid, furfural and furfural hydroxymethyl (HMF). The studies indicated that the hydrolyzate detoxified with activated carbon showed a considerable decrease in the concentration of the inhibitor compounds, reducing 91.52% HMF, 92.63% furfural, 33.48% acetic acid and 14.48% formic. Using the hydrolyzate presented growth and production of lipids of *R. mucilaginose* with 5.11 g / L and 17.26%, respectively. *R. mucilaginose* well grown with detoxified hydrolyzate in a 10 L batch reactor with a C / N ratio of 19.85 yielded a lipid yield and lipid content reached 1.78 g / L and 14.62%, respectively. High-density cell culture was studied with a two-stage nitrogen feed strategy to improve lipid content and lipid content, obtaining 4.85 g / L and 19.75%. And the last chapter, cane molasses was used as a substrate for the production of lipids using *R. glutinis*. The addition of yeast extract with ammonium sulfate and Tween 20 increased the accumulation of lipids by 27%. The optimum condition using the surface response methodology (MRS) to obtain maximum lipid concentration was with a concentration of molasses of 5.83% and 1.78 g / L of ammonium sulfate for 6 days. The lipid production was improved in a stirred tank bioreactor having a pH of 5.5, aeration rate of 1.5 vvm and 1.5 g / L with Tween 20. In the batch fermentation, it was possible to obtain a biomass production of 27 , 95 g / L, a lipidic content of 60.94% and 3.40 g/L/ day of lipid productivity, that is, under the conditions studied, increase biomass production by 5 times and increase the accumulation of lipids from of a native yeast isolated from soil**.**

Key-words: Single cell oil. New microorganisms isolated. Biodiesel. Molasses cane**.** Hydrolyzate of oil palm empty fruit bunches (OPEFB). Bioprocess optimization.

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SUMARIO

1 INTRODUCTION

The main global problem of economic, social, environmental and human survival is climate change (IPCC, 2007). Climate change is a climatic transformation caused by emissions of greenhouse gases (GHG) derived from human activities from the generation of the Industrial revolution. The Intergovernmental Panel on Climate Change (IPCC) concluded that this phenomenon began more than two centuries ago and humans contribute every day to increase it (IPCC, 2007, Zamarripa and Solis 2013). Human activities generate long-term GHG emissions: carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulfur hexafluoride (SF_6), according to the UN (1997).

The 21st session of the Conference of the Parties (COP) concluded with the adoption of a historic agreement (Paris Agreement) to combat climate change and promote measures and investments for a low carbon, resilient and sustainable future. The main aim of the universal agreement is to keep the temperature rise in this century well below two degrees Celsius, and to push efforts to limit the temperature rise even further, below 1.5 ° C above pre-industrial levels. In addition, the agreement seeks to strengthen the ability to cope with the impacts of climate change (UNFCCC, 2016).

The Paris Agreement is supported by the Lima-Paris Action Agenda (LPAA), an initiative led by France, Peru, the UN Secretary General and the Secretariat of the United Nations Framework Convention on Climate Change (UNFCCC). Its objective is to show the commitments and partnerships of cities, regions, companies and civil society organizations, often with governments, that reduce greenhouse gas emissions and build resilience against climate change.

Brazil, Argentina and Colombia are the only Latin American countries that are among the main producers of bioethanol and biodiesel in the world, according to the latest study on biofuels published by the Economic Commission for Latin America and the Caribbean (ECLAC, 2016). Brazil is the second largest producer of bioethanol in the world, with 33.2% market share, behind the United States,

responsible for 54.7% of world production, according to data from 2009. Colombia is the tenth largest producer country with 0.4%. Argentina, on the other hand, is the second largest producer of biodiesel, with 13.1% of the market, also after the United States, which leads with 14.3%. Brazil ranks fifth, with a 9.7% share. Today, the vast majority of countries in the world, including those in Latin America and the Caribbean, have some kind of policy or instrument to promote biodiesel research and development (ECLAC 2016).

In this context, it is considered relevant and necessary to explore new sources of biofuels. The oleaginous yeasts could be used as feedstock for biodiesel production due that its structure and composition is like that of common vegetable oils (Meng et al., 2007; Mesters et al., 1996; Papanikolaou and G. Aggelis, 2002; Wang and Ren, 2014). Additionally, compared to vegetable oil and animal fat, the microbial oil has many other advantages, such as less labor required, short life cycle, easier to scale up and less affection by season, venue, and climate (Li et al., 2008; Leman, 1997). Moreover, in addition to oil, oleaginous microorganisms contain significant quantities of proteins, carbohydrates, and other nutrient contents (Sanchez et al., 2003; Kot et al., 2016). Those byproducts are useable in industry, which result in reduction the production cost in biodiesel. Thus, microbial oil has been considered as one of the potential oil feedstock for biodiesel production, though there are many works associated with microbial oil need to be carried out further (Wang and Ren, 2014).

That is why, in this doctoral thesis, a study on oleaginous yeast was developed starting with the isolation, selection and identification until the development of two bioprocesses to produce microbial oil using low cost agroindustrial residues, which were the lignocellulosic residues of oil palm empty fruit bunches (OPEFB) and sugar cane molases, whose objective of the research work is to boost the development of third generation biofuels.

2 LITERATURE REVIEW

2.1 CHANGE CLIMATE

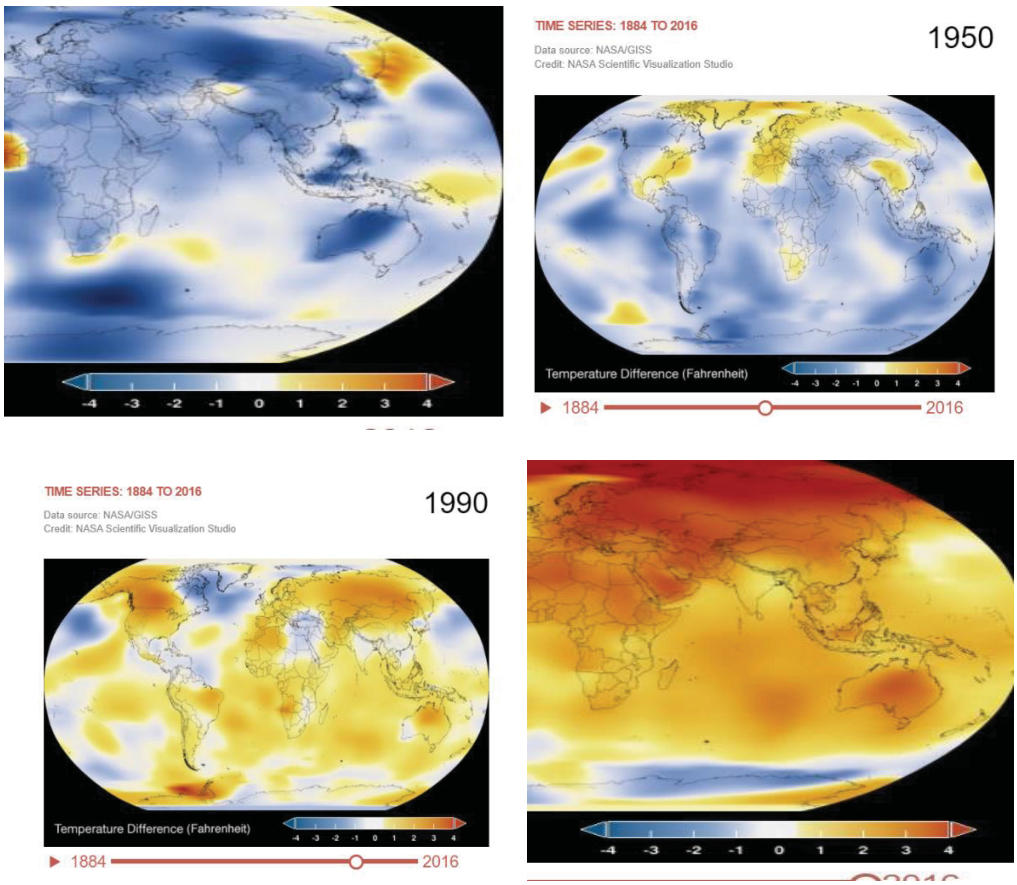
Climate change is one of the most complex issues facing us today. It involves many dimensions science, economics, society, politics and moral and ethical questions and is a global problem, felt on local scales, that will be around for decades and centuries to come. Carbon dioxide, the heat-trapping greenhouse gas that has driven recent global warming, lingers in the atmosphere for hundreds of years, and the planet (especially the oceans) takes a while to respond to warming. Despite increasing awareness of climate change, our emissions of greenhouse gases continue on a relentless rise (FIGURE 2.1). In 2013, the daily level of carbon dioxide in the atmosphere surpassed 400 parts per million for the first time in human history. The last time levels were that high was about three to five million years ago, during the Pliocene era. As atmospheric CO_2 increases over the next century, it is expected to become the first or second greatest driver of global biodiversity loss (Sala et al., 2000; Thomas et al., 2004).

FIGURE 2.1 - BASED ON THE COMPARISON OF ATMOSPHERIC SAMPLES CONTAINED IN ICE CORES AND MORE RECENT DIRECT MEASUREMENTS, PROVIDES EVIDENCE THAT ATMOSPHERIC CO_2 HAS INCREASED SINCE THE INDUSTRIAL REVOLUTION. (CREDIT: VOSTOK ICE CORE DATA/J.R. PETIT ET AL.; NOAA MAUNA LOA CO2 RECORD

FONTE: (NASA, 2017)

The average global temperature of the planet in the last 100 years has increased by 0.7° C and since 1975 the increase per decade is approximately 0.15° C. For the rest of the century, the average annual temperature will increase by 2 to 3° C, which will mean the greatest climate change in the last 10,000 years for the planet and it will be difficult for people and ecosystems to adapt to this disturbance in the climate (IPCC, 2011). Atmospheric concentrations of CO_2 have increased by 30% in the last 100 years, due to man-made combustion through fossil fuels. The steady increase in atmospheric CO_2 has been responsible for most of the warming. (FIGURE 2.2).

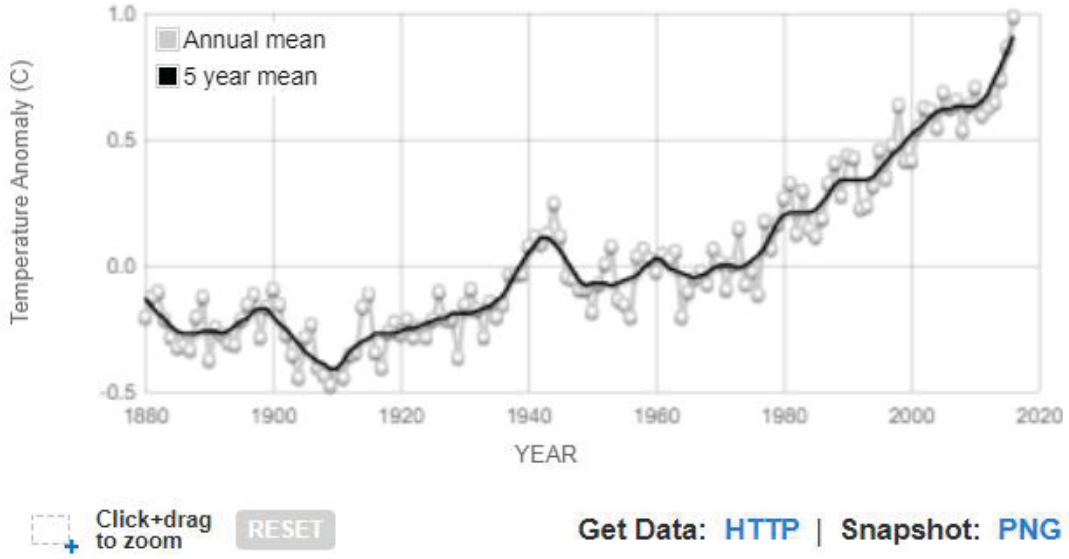
FIGURE 2.2 - CHANGE OF THE TEMPERATURE OF THE EARTH DURING THE LAST 32 YEARS



FONTE: NASA, 2017.

Most of the warming occurred in the past 35 years, with 16 of the 17 warmest years on record occurring since 2001. Not only was 2016 the warmest year on record, but eight of the 12 months that make up the year from January through September, with the exception of June were the warmest on record for those respective months (https://www.giss.nasa.gov/research/news/20170118/).

FIGURE 2.3 - CHANGE IN GLOBAL SURFACE TEMPERATURE RELATIVE TO 1951-1980.



FONTE: NASA, 2017

This graph (FIGURE 2.3) illustrates the change in global surface temperature relative to 1951-1980 average temperatures. Sixteen of the 17 warmest years in the 136-year record all have occurred since 2001, with the exception of 1998. The year 2016 ranks as the warmest on record.

In the last 40 years, anthropogenic emissions have practically doubled. Total annual anthropogenic GHG emissions by gas groups (1970-2010) were 65% CO_2 from burning fossil fuels and industrial processes, 11% CO_2 per land use change (CUS), 16% methane per Livestock, agriculture, waste, others and 8% of other gases and processes. Total emissions rose from 27 giga tonnes (Gt) of CO_2 in 1970 to 49 Gt of CO_2 in 2010 (IPCC, 2014, Lima COP20, 2014).

At the global level, annual GHG emissions are currently estimated at around 50 Gt. Average global GHG emissions per capita are seven tons. The Intergovernmental Panel on Climate Change (IPCC) predicts that to keep the temperature rise below 2 ° C by the middle of the 21st century, per capita emissions are expected to fall to two tons. Reducing emissions involves a more efficient management of energy and water resources, which would bring about a greater disposition of natural wealth for the absorption of GHG and the provision of environmental services such as biodiversity and water (CEPAL, 2015).

During the "United Nations Framework Convention" held in Kyoto, Japan, a protocol was formulated containing the mechanisms to deal comprehensively with the greenhouse effect (ONU, 1997). One of the agreements is to reduce the negative impact on the environment through more efficient processes, machines and implements and using renewable energy sources.

The protocol was initially adopted on 11 December 1997 but did not enter into force until 16 February 2005. In November 2009, there were 187 countries including Costa Rica, El Salvador, Honduras, Guatemala, Mexico, Nicaragua and Panama. Which ratified the protocol and which account for 63.7% of GHG emissions. The United States, the world's largest emitter of greenhouse gases, has not ratified the protocol (UNFCCC, 2016).

The phenomenon of climate change, population growth worldwide and consequently its increased demand for energy and changing environmental conditions, as well as the increasing reduction and difficulty of access to fossil fuels have led to Society, various government agencies, education and research centers, seek alternative sources of clean energy to meet their needs. Given this scenario, several countries in the Americas and the world are pushing for the use of cleaner and more efficient renewable energies such as biofuels to reduce the effects of climate change and contribute to environmental conservation (Zamarripa and Solís, 2013).

2.2 BIOFUELS

In the twentieth century major research emphasis was given for the development of petroleum, coal, and natural gas based refinery to exploit the cheaply available fossil feed stock. These feedstocks are used in industry to produce multiple products such as fuel, fine chemicals, pharmaceuticals, detergents, synthetic fiber, plastics, pesticides, fertilizers, lubricants, solvent, waxes, coke, asphalt, etc. to meet the growing demand of the population (Bender et al., 2000; Demirbas, 2006). Currently, the fossil resources are not regarded as sustainable and questionable from the economic, ecology and environmental point of views (Kamm et al., 2006). The burning of fossil fuels is a big contributor to increasing the level of CO2 in the atmosphere which is directly associated with global warming observed in recent decades (Naik et al., 2010). The adverse effects of greenhouse gas (GHG) emissions on the environment, together with declining petroleum reserves, have been realized. Therefore,the quest for sustainable and environmentally benign sources of energy for our industrial economies and consumer societies has become urgent in recent years (Mabee et al., 2005). Consequently, there is renewed interest in the production and use of fuels from plants or organic waste.

The biofuels produced from the renewable resources could help to minimize the fossil fuel burning and CO_2 production. Biofuels produced from biomass such as plants or organic waste could help to reduce both the world's dependence on oil and CO_2 production. These biofuels have the potential to cut CO_2 emission because the plants they are made from use CO_2 as they grow (Osamu and Carl,1989). Biofuels and bioproducts produced from plant biomass would mitigate global warming. This may due to the CO2 released in burning equals the CO_2 tied up by the plant during photosynthesis and thus does not increase the net CO_2 in the atmosphere (Naik et al., 2014)

'First generation' biofuels can offer some CO_2 benefits and can help to improve domestic energy security. But concerns exist about the sourcing of feedstocks, including the impact it may have on biodiversity and land use and

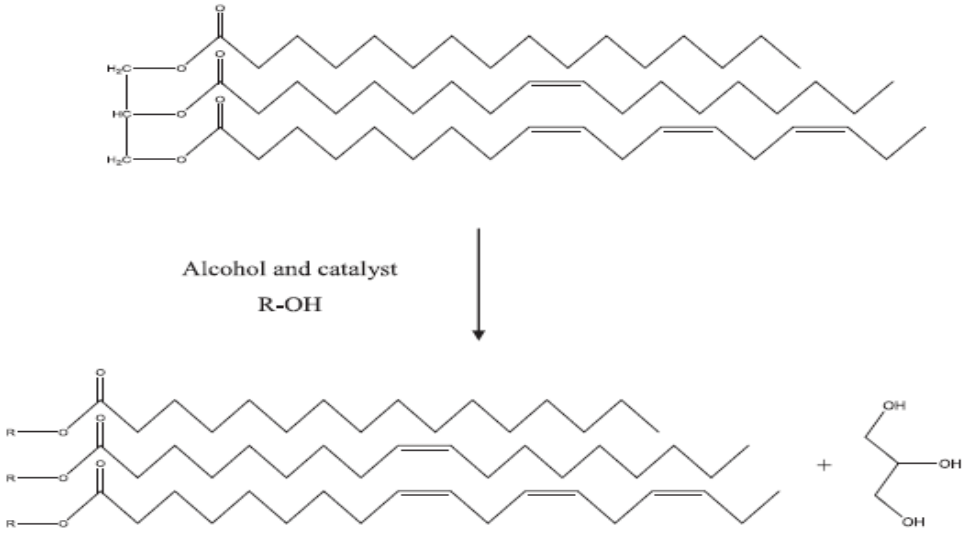
competition with food crops. A ‘first generation’ biofuel (i.e. biodiesel (bio-esters), bio-ethanol, and biogas) is characterized either by its ability to be blended with petroleum-based fuels, combusted in existing internal combustión engines, and distributed through existing infrastructure, or by the use in existing alternative vehicle technology like FFVs (‘‘Flexible Fuel Vehicle’’) or natural gas vehicles.

The production of 1st generation biofuels is commercial today, with almost 50 billion liters produced annually. There are also other niche biofuels, such as biogas which have been derived by anaerobic treatment of manure and other biomass materials. However, the volumes of biogas used for transportation are relatively small today (Naik et al., 2010) However, the first generation biofuels seems to create some skepticism to scientists. There are concerns about environmental impacts and carbon balances, which sets limits in the increasing production of biofuels of first generation. The main disadvantage of first generation biofuels is the food-versus-fuel debate, one of the reasons for rising food prices is due to the increase in the production of these fuels (Laursen, 2006). Additionally it is claimed that biodiesel is not a cost efficient emission abatement technology. Therefore, for the abatement of GHG, it is recommended to have more efficient alternatives based on both renewable and conventional technologies (Esiberg, 2006).

2.2.1 BIODIESEL

Biodiesel is produced by trans-esterification of TAG using an alcohol, either ethanol or methanol, in the presence of a base, producing fatty acid methyl- or ethyl-esters (FIGURE 2.4). Fatty acid composition has been reported to have significant impacts on the performance of biodiesel (Knothe, 2005, 2008; Steen et al., 2010). Chain length, the degree of unsaturation and branching modify the cetane number, melting point, oxidative stability, kinematic viscosity and heat of combustion, which are relevant properties that a biodiesel must meet in order to comply with official standards, such as ASTM D6751 and EN 14214 (Knothe, 2008).

FIGURA 2.4 - CONVERSION OF TRIACYLGLYCERIDES (TAG) TO FATTY ACID METHYL ESTERS (FAME) PLUS GLICEROL BY TRANSESTERIFICATION



FONTE: I.R. Sitepu et al., 2014.

The relationship between the structural features and the chemical specifications is described in Table 1. Edible plant oils used for biodiesel worldwide are rapeseed (84%), sunflower (13%), palm oil (1%), soybean and others (2%) (Atabani et al., 2012), primarily because these oils have high oleic acid content. This fatty acid provides superior ignition quality, idealmelting point, kinematic viscosity aswell as improved oxidative stability (Knothe, 2005, 2008; Steen et al., 2010).

TABLE 2.1- RELATIONSHIP BETWEEN FATTY ACID STRUCTURE AND BIODIESEL PERFORMANCE PARAMETERS. THUS IT HAS BEEN PROPOSED TO SELECT YEAST THAT PRODUCE HIGH AMOUNTS OF OLEIC ACID AS POTENTIAL CANDIDATES FOR BIODIESEL PURPOSES, BECAUSE THIS FATTY ACID BEST MEETS THESE CRITERIA.

	Cetane number (ignition quality: greater is better)	**Melting point (lower is better)**	**Oxidative stability (more stable is better)**	**Kinematic viscosity (less viscous is better)**	**Heat of combustión (greater is better)**
Chain length NR	Longer is better	Shorter is better	Shorter is better	Shorter is better	Longer is bette
Degree of unsaturation	Saturated is better	Unsaturated is better	Saturated is better	Unsaturated is better	NR
Branching	NR	Branching is better	NR	NR	NR

FONTE: KNOTHE, 2005; KNOTHE, 2008; STEEN et al., 2010, I.R. Sitepu et al., 2014.
NOTA: Not relevant.

The fatty acid profile of biodiesel derives from its precursor oil or mixture, if no intermediate winterization (i.e. improvement of cold flow properties) or selective interesterification is applied. For example, when the source is low erucic rapeseed oil (canola), the predominant fatty acids are oleic (55–65%) and linoleic (18–24%) (Hui, 1996). Two types of sunflower oil are available: high oleic (60– 65%) or high linoleic (73–78%) sunflower oil (Hui, 1996). The former is more expensive, but is preferred for biodiesel purposes due to its higher oleic content. In the case of palm oil, it is common to separate the olein fraction from the stearin fraction via winterization or other crystallization techniques. The olein fraction is preferred for production of biodiesel, due to its high oleic acid content (N40%) (Firestone, 2006). Soybean, corn, and high oleic safflower and other types of oils are rarely used for biodiesel production due to high cost and poor oleic acid content.

Total world biodiesel production is rising sharply to meet the demand: production rose from 15,000 barrels per day in 2000 to 289,000 in 2008 (Atabani et al., 2012), with highest production in the European Union and USA. Plant oil comprises a large portion of the production cost of biodiesel (Miao and Wu, 2006). The rising cost of edible plant oils, and public debate of the “food vs. fuel” issue, have encouraged development of so-called “second-generation” biodiesel from nonedible plant oils such as jatropha, jojoba, and waste oils such as cooking grease and animal fats. However, these oils may not be sufficiently abundant to meet global needs, and animal fats perform poorly in cold weather as they do not fit the specifications in TABLE 2.1.

2.2.2 Potential use of microorganisms derived microbial oil

In the review work done by I.R. Sitepu et al. (2014) mentions that “Third-generation” biodiesel is under development using oil accumulating microbes such as microalgae, bacteria, yeasts and other fungi. Single cell oils (SCO), or microbial systems that produce and store oil, have attracted significant research attention

recently, primarily in response to the rising price of petroleum. Oleaginous microbes can convert substrates such as carbon dioxide, sugars, and organic acids to SCO.

While some species produce intracellular neutral lipid continuously, most cell types require stressors like nutrient deprivation to stimulate lipid synthesis. Once the cells produce the lipid, they are harvested and the cells are lysed by solvent, mechanical, enzymatic, or other means, releasing the lipid. The lipid is then separated from the cell fraction, and the neutral lipid undergoes chemical refining to produce ester or other target molecule, by releasing the glycerol from the individual fatty acids. Typically, this is done by acid or base hydrolysis in the presence of an alcohol. While the methodology has been established, the current barrier in SCO production is developing a robust system that is cost competitive with petroleum-based fuels. This can be achieved by the development of strains able to convert low cost substrates, grow quickly to high density, and produce larger quantities of neutral lipid, and development of improved harvesting and dewatering Technologies (Sitepu et al., 2014)

Microbial oils do have some inherent advantages over plant, oils microbial biodiesel could be produced year-round (given available feedstock), on land unsuitable for agriculture, with production rates up to 100x that of plant oils in liters/hectare/year (Atabani et al., 2012). Of the 33,000 known species of algae (Brodie et al., 2007; Guiry and Cunningham, 1984), at least forty are considered oleaginous (Griffiths and Harrison, 2009), i.e. they have been demonstrated to accumulate over 20% lipid by dry weight (Ratledge, 1979). Doubling times vary considerably, depending on species and growth conditions, ranging from hours to days (Sheehan, 1998). Oil and hydrocarbon content of oleaginous species can range from 20–60%, and up to 80% for exceptional genera such as Nannochloropsis, Schizochytrium and Botryococcus (Chisti, 2007). High-value nutritional oils including docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), gamma linolenic acid (GLA) and arachidonic acid (ARA) are currently produced commercially utilizing different types of microorganisms. This demonstrates that SCO production is not limited to one clade of microorganisms (Cohen and Ratledge, 2005). For example, filamentous fungi have been used for the commercial production of GLA (*Mucor*

circinelloides) and ARA (*Mortierella alpina*). *Ulkenia sp*., a marine microalga is also being used commercially for producing DHA-rich oil (Cohen and Ratledge, 2005). The microalga Nannochloropsis is used to commercially produce EPA (Green et al., 2012; Weissman et al., 2012). Furthermore, certain Thraustochytrids, comprising species such as Schizochytrium sp., are also being used to source commercial oils rich in DHA. These organisms in the past were originally classified as fungi because of their heterotrophic nature and superficial resemblance to chytrids, but recently they were reclassified as microalgae-like microorganisms by molecular biology techniques. *Dinoflagellates* such as *Crypthecodinium cohnii*, are also being used commercially to source high DHA oil (Cohen and Ratledge, 2005; Eckelberry and Green, 2010; Ratledge, 1993).

Commercial production of these oils demonstrates that large-scale production of microbial oil is feasible, given an appropriate process and market. However, these examples are high value specialty chemicals. Further strain and process improvements are needed to make production of microbial commodity oleochemicals such as biodiesel economically feasible. Estimating the cost of single cell oils has been difficult, and biodiesel cost projections have ranged from $1 to $80 per gallon (Davis et al., 2011). The economics of algal SCO has been examined more thoroughly than other cell types, and provides a template for other SCO biofuels. Algae, unlike yeast or bacterial models, have different culturing methodologies that add additional dimensions to the economic analysis.

Improvements in the SCO production process could result in an economically viable process. These include reduction of feedstock transport, pretreatment and hydrolysis costs; reduction of aeration and pH control costs; reduction of contamination potential; year-round production; improved utilization of the carbohydrates in lignocellulosic hydrolysates; faster microbial growth to a higher cell density; higher oil accumulation; improved oil harvesting technologies; conversion of residual yeast cell mass into valuable coproducts; and reduced waste disposal costs. In order for single cell derived biodiesel to become economically viable, the biodiesel must be viewed as one member of a portfolio of high value coproducts. Prevailing models instead view SCO as one valorized product, with the rest of the yeast cell

mass considered as simply a waste product, or low-profit product streams such as animal feed or fertilizer. For comparison, current business models in oilseed processing have met significant success in generating multiple coproducts, with a significant proportion of revenue coming from coproducts such as texturized protein, lecithin and sterols (Sitepu et al., 2014)

Since microbial lipids can be produced using various waste streams (e.g. whey, lignocellulosic hydrolysates) as substrate, it has been considered a potential feedstock to support a sustainable biodiesel industry. In fact, industrial waste and lignocellulosic material may be used to provide sugar and nitrogen sources to produce lipids, thus showing good characteristics such as short life cycle and low affection by climate (Amaretti et al., 2010). Koutinas et al. (2014) reported that the unitary cost of purified microbial oil production from glucose is $3.4/kg for an annual production capacity of 10,000 t microbial oil. The cost of microbial oil production and conversion to biodiesel was estimated at $5.5/kg oil and $5.9/kg biodiesel, accordingly, when a cost of glucose of $400/t was assumed. Koutinas et al. projected that if the cost of manufacture of microbial oil is reduced by 50% until the next decade then microbial oil could potentially compete with vegetable oils regarding biodiesel production cost. In most cases, the cost of microbial oil production from oleaginous yeast is lower than the cost of oil production from algae.However, oil production algae and yeast should be developed and compared further in order to evaluate their potential for biodiesel production (Koutinas et al., 2014). Therefore, the production of microbial oil using cheap substrates, such as waste streams, is a key parameter in order to develop na economically and environmentally viable biodiesel production process (Thiru et al., 2011).

2.3 OLEAGINOUS YEASTS

Single cell oils (SCOs) are intracellular storage lipids comprising of triacyglycerols (TAGs). SCOs are produced by oleaginous microorganisms which are able to accumulate between 20% and up to 80% lipid per dry biomass in the stationary growth phase under nutrient limitations, e.g., nitrogen or phosphor, with simultaneous excess of carbon source. Depending on the oleaginous microorganism

including bacterial, yeast, microalgae or fungal species, fatty acid profile of SCOs can vary making them highly suitable for diverse industrial applications (Ochsenreither et al., 2016).

TABLE 2.2 - OIL CONTENT OF SOME MICROORGANISMS

Microorganisms	Oil content (% dry wt)	Microorganisms	Oil content (% dry wt)
Yeast		**Bacterium**	
Candida curvata	58	Arthrobacter sp.	40
Rhodosporidium toruloides	50	Acinetobacter calcoaceticus	27 – 38
Yarrowya Lipolytica	44	Rhodococcus opacus	24 – 25
Rodotorulha glutinis	17.8 – 66	Bacillus alcalophilus	18 – 24
Microalgae		**Fungi**	
Botryococcus braunii	25 – 70	Aspergillus oryzae	57
Cylindrotheca sp	16 – 37	Mortierella isabellina	40
Nitzschia sp. 45–47	45 – 47	Humicola lanuginosa	75

FONTE: Adaptaded and modifidied from Meng et al., 2009.

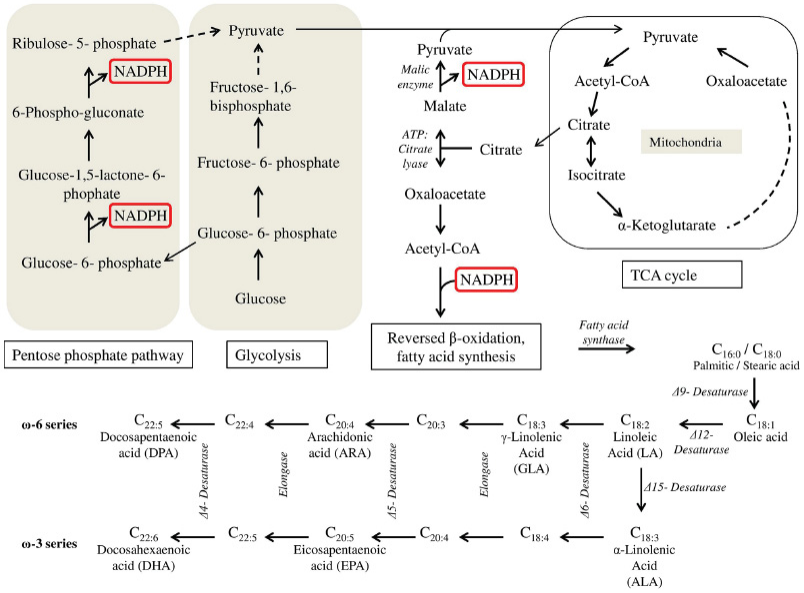
Considering the foreseeable depletion of crude oil, the highly controversial “food-or-fuel” discussion about using plant oils for biodiesel production, overfishing of the oceans and the urgent need for the reduction of greenhouse gas emissions, microbial SCOs seems to be intriguing substitutes for crude, plant, and fish oil. Furthermore, microbial lipid production is independent from season, climate, and location, can be realized using a wide range of carbon source, e.g., waste streams from food industry or renewable carbon sources, in case of microalgae even from CO_2, does not use arable land, results in high yields and can be accomplished with genetically modified organisms changing fatty acid composition and enhancing yields. Whereas the production of very long polyunsaturated fatty acids, i.e., docosahexaenoic acid (DHA; 22:6, ω-3), arachidonic acid (ARA; 20:4, ω -6) and oleic acid (18:1, ω-9), are commercialized using the oleaginous fungus Mortierella alpina, yeast Rodotorula glutinis and different oleaginous microalgae (for an overview see (Ratledge, 2004; Kot et al., 2016).

2.3.1 MECHANISM OF LIPID BIOSYNTHESIS

The ability of (eukaryotic) oleaginous organisms to accumulate large amount of lipids is not accounted to a difference in fatty acid biosynthesis compared to non-oleaginous species. However, a continuous supply of acetyl-CoA and NADPH for the fatty acid production by a reversed b-oxidation has to be assured under nutriente limited but carbon excess conditions. The continuous production of acetyl-CoA in oleaginous microorganisms is achieved by a cascade of enzyme reactions triggered by a nutrient limitation (in biotechnology, usually a nitrogen limitation is used) leading essentially to a citrate accumulation in the mitochondria. A unique feature of oleaginous organisms is the AMP-dependency of isocitrate dehydrogenase, an enzyme of the TCA cycle catalyzing the oxidative decarboxylation of isocitrate. In case of a nitrogen limitation, the activity of AMP deaminase, catalyzing the cleavage of AMP to IMP and ammonia, is increased considerably due to the nitrogen limitation leading to low AMP levels inside the mitochondria. As a consequence, isocitrate is not further metabolized and converted to citrate by the enzyme aconitase. Citrate is transported into the cytosol and cleaved by the enzyme ATP:citrate lyase to acetyl-CoA and oxaloacetate leading eventually to the continuous supply of acetyl-CoA for fatty acid synthesis. ATP:citrate lyase was found so far in all reported oleaginous microorganisms, however, in some non-oleaginous organisms the enzyme is also presente **(**Botham and Ratledge, 1979; Boulton and ratledge, 1981a,b; Evans and Ratledge, 1984; Wynn et al., 2001; Ratledge, 2002**).** Besides acetyl-CoA a lot of reducing power in form of NADPH is necessary for the production of fatty acids, i.e., 16 moles of NADPH for the synthesis of stearic acid (C18). Although not finally clarified yet, malic enzyme is discussed to be mainly responsible for NADPH supply. Malic enzyme catalyzes the decarboxylation of malate (resulting from oxaloacetate) to pyruvate which is transported into the mitochondria. However, as malic enzyme activity has been reported not to be involved in NADPH regeneration in some oleaginous organisms, e.g., Yarrowia lipolytica (Zhang et al., 2013), alternative routes may also be responsible. Additionally, NADPH regeneration via the pentose

phosphate pathway is also an option (Tang et al., 2015; Zhao et al., 2015). An overview about the biosynthesis is given in FIGURE 2.5.

FIGURE 2.5 - BIOSYNTHESIS OF FATTY ACIDS UNDER SCO PRODUCING CONDITIONS IN OLEAGINOUS EUKARYOTIC MICROORGANISMS.



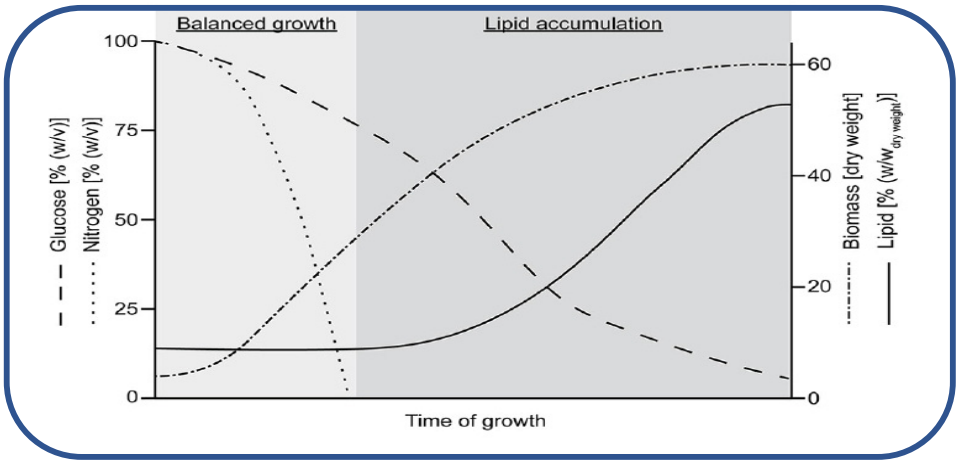
FONTE: Chemler et al., 2006; Tang et al., 2015)

The described fatty acid biosynthesis ends in almost every organism with the formation of palmitic (16:0) or stearic (18:0) acid. For the production of the especially desired polyunsaturated fatty acids a subsequent series of elongation and desaturation by elongases and desaturases, respectively, is necessary. Therefore, the potential of amount and type of produced PUFA is dependente on the genes of elongases and desaturases present in the genome of the respective oleaginous organism. During the last years and decades many studies and reviews dealing with SCO production have occurred. Since the commercialization of SCO production besides the mentioned PUFA production is still uneconomical, more and more researchers are focusing now on combined approaches of genetic engineering to enhance yields and productivity and the usage of low-cost substrates. However, a holistic assessment of the processes including downstream processing is often missing which is orientated on industrial scale and subsequente application of the oil (Ochsenreither et al., 2016).

2.3.2 MICROORGANISMS FOR SINGLE CELL OILS PRODUCTION

Lipids and oil are produced by all living macro- and microorganisms for essential structural and functional roles such as the formation of permeable membranes of cells and organelles in the form of a lipid bilayer (Dowhan and Bogdanov, 2013). However, only a relatively small number of microorganisms are able to accumulate amounts of cellular lipids over 20 or even up to 80% of their cell mass as a reserve storage material. These are termed as oleaginous microorganisms (Ratledge, 2004). The microbial production of SCO offers several advantages compared to the use of animal or plant sources. The cultivation of microorganisms is independent from geographic or climatic constraints, has short producing periods and several substrates, including industrial wastes, can be used (Ward and Singh, 2005b; Li Q. et al., 2008). The main producers of lipids are fungi, yeasts, and algae, while bacteria are bad producers (Wynn and Ratledge, 2005; Li Y. et al., 2008; Bellou et al., 2016). The lipid accumulation as a reserve storage is triggered by an excess of carbón source and one limiting nutrient, usually nitrogen. Under these conditions the carbon flux is directly channeled toward lipid synthesis and discrete oil droplets consisting of triacylglycerols are formed within the cells (Ratledge, 2004; Wynn and Ratledge, 2005). The typical course of lipid accumulation by oleaginous microorganisms is shown in FIGURE 2.6.

FIGURE 2.6 - TYPICAL COURSE OF LIPID ACCUMULATION BY OLEAGINOUS MICROORGANISMS



FONTE: Wynn and Ratledge, 2005.

The first phase of balanced growth, where all nutrients are in excess is characterized by the production of biomass and the consumption of the carbon and nitrogen source. If the nitrogen is exhausted, the biomass production is reduced and the accumulation of lipid starts (Wynn and Ratledge, 2005). In contrast, non-oleaginous microorganisms would either stop cell division or accumulate polysaccharides, including glycogens and various mannans, glucans etc. (Ratledge, 2004). An overview on the microorganisms used for SCO production and the range of produced cellular lipids in their dried biomass is given in TABLE 2.3. High amounts of cellular lipids are produced by microorganisms belonging to the genera *Cryptococcus, Cunninghamella, Mortierella* and *Rodotorula*. The genus *Mortierella* is capable to produce SCO with a unique composition, containing high amounts of PUFAs (Asadi et al., 2015). M. alpina is used in an industrial process for the production of arachidonic acid (ARA, 20:4, &-6) for food supplementation by DSM (Béligon et al., 2016). The genus Rodotrula can be used in the industrial process for the production biodiesel because they are rich in oleic acid.

TABLE 2.3 - OVERVIEW OF THE GENERA USED FOR THE PRODUCTION OF SINGLE CELL OIL (SCO) AND AMOUNTS OF CELLULAR LIPIDS ACCUMULATED PER DRY WEIGHT

(continua)

Kingdom	Division	Order	Genus	SCO [%(w/wDW)]	References
Chromalveolata	Heterokontophyta	Labyrinthuales	Aurantiochytrium	65	Huang et al., 2012
			Schizochytrium	49-67	Chang et al., 2013; Ling et al., 2015
		Phyitales	Pythium	76	Cheng et al., 1999
Fungi	Ascomycota	Eurotiales	Aspergillus	18	Lin et al., 2010
		Saccharaomycetales	Candida	2-27	Chatzifragkou et al., 2011
			Yarrowia	7-43	Papanikolaou and Aggelis, 2002; Chatzifragkou et al., 2011.
			Zygosaccharomyces	13	Chatzifragkou et al., 2011
	Zygomycota	Mucorales	Cunninghamella	21 - 78	Gema et al., 2002; Fakas et al., 2009b; Chatzifragkou et al., 2010, 2011
			Mucor	18	Chatzifragkou et al., 2011
			Thamnidium	43	Chatzifragkou et al., 2011

			Zygorhynchus	42	Chatzifragkou et al., 2011

TABLE 2.3 - OVERVIEW OF THE GENERA USED FOR THE PRODUCTION OF SINGLE CELL OIL (SCO) AND AMOUNTS OF CELLULAR LIPIDS ACCUMULATED PER DRY WEIGHT

(conclusão)

Kingdom	Division	Order	Genus	SCO [%(w/wDW)]	References
Fungi	Basidiomycota	Sporidiales	Rhodotorula	22- 52	Zhao et al., 2010; Chatzifragkou et al., 2011,
		Sporidiobolales	Sporobolomyces	30 - 50	Matsui et al., 2011
	Zygomycota	Tremellales Ustilaginales	Cryptococcus	33 - 78	El-Fadaly et al., 2009; Chi et al., 2011
			Rhodosporidium	33	Matsakas et al., 2015
		Mucorales	Cunninghamella	21 - 78	Gema et al., 2002; Fakas et al., 2009b; Chatzifragkou et al., 2010, 2011
			Mucor 18	18	Chatzifragkou et al., 2011
			Thamnidium	43	Chatzifragkou et al., 2011
			Zygorhynchus	42	Chatzifragkou et al., 2011
		Mortierellas	Mortierella	5 - 74	Bajpai et al., 1991; Fakas et al., 2009b; Chatzifragkou et al., 2010; Economou et al., 2011a; Gao et al., 2013; Stressler et al., 2013; Zeng et al., 2013

FONTE: Ochsenreither et al. 2016.

2.3.3 Processes for the microbial production

The microbial production of SCO can be either conducted as submerged (SmF) or solid state fermentation (SSF). Table 2.4 summarizes culture conditions for SCO production by SmF and the cellular lipid contents obtained. The amount of lipids accumulated mainly depends on the mode of cultivation, the carbon and nitrogen source, pH and temperature.

The most frequently used carbon source is glucose. Various mono- or disaccharides and carboxymethylcellulose (CMC) were tested as carbon source for Mortierella isabellina by Zeng et al. (2013). In this study cellular lipid contents above 60% were generated with xylose, glucose and fructose as substrates. CMC was a poor substrate, implying the absence of a cellulase system (Zeng et al., 2013). Cultivation of Cunninghamella echinulata and M. isabellina showed that the carbon source is a crucial parameter for the production of SCO as well as g-linolenic acid (GLA, 18:3, &-6). Both fungi showed satisfactory growth on glucose, fructose, and

molasse, while M. isabellina failed to grow on saccharose (Chatzifragkou et al., 2010). Due to the high amounts of carbón source necessary to trigger the lipid accumulation it is economically effective to use low-cost raw materials, such as glycerol (Fakas et al., 2009b; Chatzifragkou et al., 2011; Tchakouteu et al., 2015), commercial sugars (Chatzifragkou et al., 2010), plant material (Lin et al., 2010; Economou et al., 2011b; Zeng et al., 2013; Matsakas et al., 2015), and lignocellulosic materials (Zeng et al., 2013). Glycerol, a waste product of the biodiesel production was tested for example by (Chatzifragkou et al., 2011) as a carbon source for 15 eukaryotic microorganisms. The tested yeasts accumulated up to 22% (w/w) lipids. On the contrary, the tested fungi showed cellular lipid contents of 18 to 43% (w/w). No difference between the oil accumulation of C. echinulata and M. isabellina were observed when either using raw glycerol or pure glycerol as carbon source. The successful application of plant wastes like tomato, potato or orange peels was shown by El-Fadaly et al. (2009), Gema et al. (2002), Zhao et al. (2010) resulting in cellular lipid concentrations 50%.

Besides the selection of a suitable carbon source, the nitrogen source influences the accumulation of SCO. As well organic and inorganic nitrogen sources are used individually or in combination in the literature. These include yeast extract, urea, peptone, glycine, KNO_3, NH_4NO_3, and $(NH_4)_2SO_4$ (compare table 2.4). Gao et al. (2013) investigated the influence of the nitrogen source when cultivating M. isabellina on xylose. The highest lipid accumulation (64.2%) was achieved with yeast extract. The influence of nitrogen compounds from tomato waste hydrolysate on the uptake of glucose was shown by Fakas et al. (2008). The removal of some quantities of organic nitrogen resulted in reduced glucose uptake and large amounts of biomass with low lipid content. The lipid accumulation was not effected when using glycerol as carbon source (Fakas et al., 2008). In addition to the selection of a suitable nitrogen source, the C/N ratio influences the lipid accumulation. Reported ratios range from 35 to 340 mol mol^{-1} (Papanikolaou et al., 2004; Fakas et al., 2009b; Ruan et al., 2012).

In principal, oleaginous microorganisms can be cultivated as batch, fed-batch or continuous cultures. Most of the reported experiments in literature are based on batch shaking flask cultivations (compare TABLE 2.4). A comparison of baffeled and unbaffeled flasks for *Schizochytrium sp.* showed that the use of baffled flaks increases the biomass production, the lipid accumulation and the concentration of docosahexaenoic acid (DHA, 22:6) (Ling et al., 2015). The cultivation of *M. alpina* in a stirred tank reactor resulted in an increase of lipid accumulated in the cells compared to shaking flasks (Stressler et al., 2013). In contrast, the lipid accumulation of *M. isabellina* was not affected by using a stirred tank reactor or shaking flasks, only the biomass production increased (Chatzifragkou et al., 2010).

An interesting feasibility study of an integrated process combining a heterotrophic cultivation of yeast with CO_2 recycling in a phototrophic process was published by Dillschneider et al. (2014). The oleaginous yeast *Cryptococcus curvatus* and the oleaginous microalgae *Phaeodactylum tricornutum* were used and both processes revealed in lipid contents of about 40–45% (w/w).

TABLE 2.4. SMF FOR THE PRODUCTION OF SCO: SPECIES, LIPID CONTENT, DRY WEIGHT OF BIOMASS (DW), AND CULTIVATION CONDITIONS.

(continua)

Species	SCO [% (w/wDW)]	DW [g L−1]	Cultivation mode	Carbon source	Nitrogen source	pH [-]	Temperature [◦C]	Duration	SCO composition	References
Aspergillus oryzae A-4	18.2	4.3	Batch, flask	Glucose + cellulose	Yeast extract + (NH4)2SO4	5.5	30	360 h	27.7% PUFA	Lin et al., 2010
Aurantiochytrium limacinum SR21	65.2	61.7	Fedbatch, STR	Glycerol	Yeast extract + peptone	6.8 – 7.2	22	192 h	66.3 - 87.9% PUFA	Huang et al., 2012
Candida pulcherrima LFMB 1	11.5	7.3	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 - 6	28	63	16.5 % PUFA	Chatzifragkou et al., 2011
Candida boidinii ATTC 32195	27.2	1.3	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 - 6	28	111	15.6 % PUFA	Chatzifragkou et al., 2011
Candida tropicalis	25	6.44	Batch, flask	Hemicellulose OF Palm empty fruit bunch	(NH4)2SO4	6	30	72	57.27 % PUFA	Tampitak et al., 2015
Cryptococcus curvatus NRRL-Y 1511	64	2.5	Batch, flask	Sugar cane molasses	NaNO3	4.9 - 6	28	72	-	El-Fadaly et al., 2009
Cryptococcus curvatus ATCC 20509	75	16.8	Fedbatch, STR	Hydrogen prduction effluent + acetic acid	NH4Cl	7	30	192	15.6 % PUFA	Chi et al., 2011
Cunninghamella echinulata ATHUM 4411	57.7	7.8	Batch, flask	Xylose	Yeast extract + (NH4)2SO4	5.2 - 6	28	192	6.6 % GLA	Fakas et al., 2009b
	32	12.1	Batch, flask	Molasse	Yeast extract + (NH4)2SO4	5.2 - 6	28	358	27.4 PUFA	Chatzifragkou et al., 2010
Mortierella alpina	49	-	Batch, STR	Glucose	Yeast extract	-	20	312	38 ARA	Stressler et al., 2013
	27.3	16.5	Batch, flask	Glucose	Yeast extract	-		240	63 % PUFA 38 % ARA	Stressler et al., 2013

FONTE: Adaptaded and modifidied from Ochsenreither et al. 2016.

TABLE 2.4. SMF FOR THE PRODUCTION OF SCO: SPECIES, LIPID CONTENT, DRY WEIGHT OF BIOMASS (DW), AND CULTIVATION CONDITIONS.

(continua)

Species	SCO [% (w/wDW)]	DW [g L−1]	Cultivation mode	Carbon source	Nitrogen source	pH [-]	Temperature [◦C]	Duration	SCO composition	References
Mortierella isabellina ATHUM 2935	65.5	8.7	Batch, flask	Xylose	Yeast extract + (NH4)2SO4	5.2 – 6.3	28	216	3.9 GLA	Fakas et al., 2009b
Mortierella isabellina NRRL 1757	34	12.6	Batch, flask	Straw hydrolysate	Yeast extract + (NH4)2SO5	5.5	28	360	11.6 % PUFA	Zeng et al., 2013
Mortierella ramanniana MUCL 9235	37.1	7.3	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 - 6	28	216	22.4 % PUFA	Chatzifragkou et al., 2011
Mucor sp. LGAM 365	18.1	5.3	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 - 6	28	237	31.8 % PUFA	Chatzifragkou et al., 2011
Pythium irregular ATCC 10951	76	35	Batch, flask	Glucose + Soybean oil	(NH4)2SO4	6	18	240	2 % EPA, 1 % ARA	Cheng et al., 1999
Rhodosporidium Toruloides DEBB 5533	50.86	40.3	FedBatch, STR	Sugar cane juice	Urea	4.9 - 6	28	45	71.7 % PUFA	Soccol et al 2017
Rhodosporidium toruloides CCT 0783	33	40	Batch, flask	Sorghum	-	-	30	250	-	Matsakas et al., 2015
Rhodotorula TJY15a	52.2	19.5	Fedbatch, STR	Artichoke tuber extract hydrolysate	Yeast extract + (NH4)2SO4	6	30	108	11.3 % PUFA	Zhao et al., 2010
	48.6	14.5	Batch, flask	Artichoke tuber extract hydrolysate	Yeast extract + (NH4)2SO4	6	28	108	11.3 PUFA	Zhao et al., 2010
Rhodotorula sp. LFMB	22	8	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 -6	28	168	12.4 PUFA	Chatzifragkou et al., 2011
Rhotorula glutinis ATCC204091	47.2	70.8	Fed-batch	Corncob hydrolysate	(NH4)2SO4	6	30	72	61.2 % PUFA	Liu et al. (2015)

FONTE: Adaptaded and modifidied from Ochsenreither et al. 2016.

TABLE 2.4. SMF FOR THE PRODUCTION OF SCO: SPECIES, LIPID CONTENT, DRY WEIGHT OF BIOMASS (DW), AND CULTIVATION CONDITIONS.

(conclusão)

Species	SCO [% (w/wDW)]	DW [g L−1]	Cultivation mode	Carbon source	Nitrogen source	pH [-]	Temperature [◦C]	Duration	SCO composition	References
Rhotorula glutinis ATCC204091	47.2	70.8	Fed-batch	Corncob hydrolysate	(NH4)2SO4	6	30	72	61.2 % PUFA	Liu et al., 2015
Rhotorula glutinis TISTR 5159	60.7		Fed-batch	Glycerol	NH4)2SO4					Saenge et al. 2011b
Schizochytrium sp. LU310	67.3	60.8	Batch, flask	Glucose	Corn steep powder + MSG	6.5	28	120	41 % DHA	Ling et al., 2015
Schizochytrium sp. S31	49.1	40	Batch, flask	Glycerol	Yeast extract + (NH4)2SO4	6.8	28	80		Chang et al., 2013
Thamnidium elegans CCF 1465 4	42.6	6.8	Batch, flask	Waste gycerol	Yeast extract + (NH4)2SO4	5 - 6	28	271	15.7 PUFA	Chatzifragkou et al., 2011
Yarrowia lipolytica LGAM S(7)1	43.0	8.1	Continous, STR	Glycerol	Yeast extract + (NH4)2SO4	6	28	-	-	Papanikolaou and Aggelis, 2002

FONTE: Adaptaded and modifidied from Ochsenreither et al. 2016.

2.3.4 History of oleaginous yeast

Yeasts are defined as “fungi that asexually reproduce by budding or fission, which results in growth that is comprised mainly of single cells” (Kurtzman et al., 2011b). This definition recognizes that many yeast species are capable of multicellular, mycelial growth, a characteristic that is rarely mentioned in discussions of oleaginous yeasts (Wassef, 1977). They are taxonomically diverse, belonging to two of the seven fungal phyla, Ascomycota and Basidiomycota. There are over 1600 known species of yeasts, a figure that has more tan doubled in the last 12 years (Kurtzman and Fell, 2000; Kurtzman et al., 2011a). This boom in novel yeast species descriptions has allowed discoveries in other fields such as identification and characterization of pathogenic (Cooper, 2011) or food spoilage yeasts (Fleet, 2011), profiling food and beverage fermentations (Bokulich, 2012; Bokulich and Mills, 2013; Bokulich et al., 2011, 2012a, 2012b), and biotechnology applications (Johnson and Echevarri- Erasun, 2011).

Yeasts have been harnessed for biotechnological applications for thousands of years: the yeast Saccharomyces cerevisiae, used for producing beer, bread, wine and other delightful products, has been called the “first domesticated organism” (Vaughan Martini and Martini, 1995). Yeasts are used in biotechnology applications because they are genetically tractable, easy to cultivate, have a long history of safe use, and relatively few yeast species are known to be pathogenic to humans, plants or animals. Yeasts including *S. cerevisiae* and *Pichia pastoris* are used as hosts for production of recombinant proteins in pharmaceutical and other industries. Yeasts are also particularly useful for renewable, sustainable production of biodegradable oils for food, feed, fuels and chemicals. Many discoveries were made during the early decades of yeast oil work that have propelled modern studies, including discovery of high-oil species, culture conditions that promote lipid accumulation, conversion of waste products to yeast oil, and pilot and commercial scale production of marketable products (Sitepu et. al., 2014)

The lipids of yeasts have been a subject of study since 1878 (Nageli and Loew, 1878), when the fatty acid composition of *S. cerevisiae* was reported. In 1899, Lindner observed that *Torula pulcherrima* (nowcalled *Metschnikowia pulcherrima*) could accumulate high levels of intracelular fat (Woodbine, 1959). Research on *Oidium lactis* (now called Galactomyces candidus) including fatty acid identification and quantification was performed by Kaufmann and Schmidt in Germany in the late 1930s (Piskur, 1939). Lindner observed formation of fat globules in T. pulcherrima (now called M. pulcherrima) in 1889 (Woodbine, 1959). Researchers at German research institutions including Mannheim–Waldhof intensively studied fat-forming yeasts, particularly during World Wars I and II. Early discoveries also included identification of high-oil producing yeast species such as *Lipomyces lipofera* (Nilsson et al., 1943), *Lipomyces starkeyi* (Lodder and Kreger-van Rij, 1952; Starkey, 1946), and *Rhodotorula glutinis* (Enebo et al., 1944).

A 1946 report from the British Intelligence (Stanier, 1946) based on interviews of German scientists describes work done in Germany during WorldWar II on a variety of microbial products including fats for food, feed and fuel uses. Researchers at the Mikrobiologisches Institut at Göttingen performed a systematic investigation of numerous microbes, and discovered that certain strains of yeasts accumulated high levels of intracellular lipids. Strains of Nectaromyces reukaufii (now called Metschnikowia gruessii) had production advantages including ability to grow in lab medium containing waste products as growth nutrients (whey and bran), fast growth, and early lipid accumulation (34% oil by dry weight at four days growth).

2.3.5 LIPID CONTENT COMPOSITION OF OLEAGINOUS YEASTS

Lipids are structurally diverse biological molecules that are relatively insoluble in water and soluble in organic solvents. They include two basic categories: (1) molecules derived fromisoprene units, such as steroids and carotenoids, and (2) fatty acids and their long-chain relatives such as fatty alcohols, alkanes and alkenes. Commonly used fats and oils from plants and animals belong to the latter category.

Yeasts contain various types of lipids (Blagović et al., 2001; Lösel, 1988; Rattray, 1988; Schweizer, 2004), including triacylglycerols, diacylglycerols, monoacylgycerols, fatty acids, steryl esters, free sterols, glycerophospholipids, cardiolipins, sphingolipids, glycolipids, hydrocarbons (comprising straight chain hydrocarbons, squalene and carotenoids), long chain alcohols, waxes, polyprenols, isoprenoid quinones, and others. In the plasma membrane four types of lipids can be easily distinguished: glycerophospholipids, sphingolipids, steryl esters (mainly from ergosterol), and mono and diacylglycerols. In *S. cerevisiae* the most abundant glycerophospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Schweizer, 2004). Lipid composition in the plasma membrane varies among strains, even closely related strains (Henderson et al., 2011, 2012), and is a function of temperatura (Henderson et al., 2013b). Lipid composition is highly correlated with cell function such as ethanol tolerance (Henderson et al., 2013a).

Oleaginous yeasts store lipids mainly in the form of triacylglycerols (TAG) in intracellular lipid bodies. The primary component fatty acids in these TAG have been under investigation for over 100 years: in 1878, ten years before the first yeast species was named, brewing yeast (S. cerevisiae) was reported to contain oleic, stearic and palmitic acid (Nageli and Loew, 1878). These three fatty acids, along with linoleic, linolenic and palmitoleic acids, are the major fatty acids of most yeast species analyzed (for example, Henderson et al., 2011; Kaneko et al., 1976; Sitepu et al., 2013; Viljoen et al., 1986; Woodbine, 1959). The dominant fatty acids in yeast storage lipids are thus similar to those found in plant-derived oils,making theman appropriate substitute for vegetable oils for biodiesel and other oleochemicals. As discussed in the Fatty acid composition of oleaginous yeasts section, fatty acid methyl ester (FAME) analysis has been used to characterize hundreds of yeast species because FAME profiles were used to identify yeast species (Augustyn et al., 1992; Botha and Kock, 1993; Jeffery et al., 1997; Van Rensburg et al., 1995; Viljoen et al., 1986) before ribosomal DNA sequencing gained prominence. However, the number of described yeast species has more than doubled since this method was commonly used, thus the reference databases no longer contain a large proportion

of known yeast species. Growth conditions must be tightly controlled when using this method for species identification, as the relative abundance of fatty acids for a given strain varies under different growth conditions, such as carbon source (Easterling et al., 2009), nitrogen depletion and age of culture (Sitepu et al., 2013). Furthermore, the fatty acid total content and relative abundance varies among strains of the same species (Kaneko et al., 1976; Leiva-Candia et al., 2014; Sitepu et al., 2012; Sitepu et al., 2013; Viljoen et al., 1986).

2.3.6 Fatty acid composition of oleaginous yeasts

The storage lipids that accumulate in oleaginous yeasts are primarily diacylglycerols (DAG) and triacyl-glycerols (TAG). The fatty acid compositions of many yeast species have been reported (Augustyn et al., 1992; Botha and Kock, 1993; Jeffery et al., 1997; Sitepu et al., 2013; Van Rensburg et al., 1995; Viljoen et al., 1986). Prior to development of ribosomal sequencing methods, fatty acid profiling was one of the methods used to identify yeast species (Augustyn et al., 1992; El Manyawi and Wogerbauer, 2000; Kock et al., 1985). In fact, companies such as MIDI Labs (bhttp://www.midilabs.comN) still offer microbe identification service based on fatty acid profile. To generate a reference database for species identification, the lipid content of hundreds of yeast species has been characterized, for example (El Manyawi and Wogerbauer, 2000; Kaneko et al., 1976; Ratledge and Wilkinson, 1988).

The fatty acid composition of intracellular TAGs is variable, and depends on the species of oleaginous yeast, growth phase, environmental conditions, and substrate and mediumcomponents. The fatty acid composition can fluctuate from strain to strain (Ratledge and Wilkinson, 1988; Sitepu et al., 2012; Sitepu et al., 2013). As much as 80–90% of the neutral lipids are comprised of TAG (Connor and Atsumi, 2010; Liu et al., 2009; Meesters et al., 1996; Radulovic et al., 2013). Each fatty acid (FA) chain is 14–20 carbons in length bound to a glycerol backbone. The most common fatty acids are 16 or 18 carbons in length (C16 and C18, respectively), either saturated (no double bonds, such as C16:0) or monounsaturated (one double

bond, such as C18:1). The most common FA of oleaginous yeasts are C18:1 (oleic acid), C16:0 (palmitic acid), and C18:0 (stearic acid) (Meesters et al., 1996). These are also the dominant FAs of plant oils used for production of biodiesel, such as canola and sunflower oil (Ageitos et al., 2011; Rattray, 1988; Woodbine, 1959). Production of an oleo chemical such as biodiesel using oleaginous yeasts therefore requires selection of a yeast species, and strain of that species, capable of producing the desired fatty acids, as well as identification of appropriate culture conditions. In most oleaginous yeast species, protein synthesis occurs while nitrogen is available, then lipid accumulation dominates when carbon is in excess (Lundin, 1950; Woodbine, 1959). During exponential growth phase, the C18:2 FA occurs in higher quantities, but decreases by stationary phase. The C18:0 and C18:1 FA becomemore prominent in stationary phase, and the addition of the double-bond in from C18:0 transitioning to the C18:1 occurs by the Δ9 desaturase (Hassan et al., 1993; Meesters et al., 1996; Ykema et al., 1988).

The fact that the fatty acid composition varies among oleaginous yeast species, among strains of the same species, and with the growth conditions has been known for many decades (Rattray, 1988; Rattray et al., 1975; Viljoen et al., 1986;Woodbine, 1959). To quantify this effect in newly discovered as well as previously known oleaginous yeast species, Sitepu et al. (2013) cultured 69 oleaginous and non-oleaginous yeasts in nitrogen-depleted defined medium with excessive glucose (120 g/L), and analyzed the fatty acid profile using GC-FID. The major fatty acids in most of the yeast strains analyzed were oleic (18:1ω9), palmitic (16:0), stearic (18:0), and linoleic (18:2ω6) acids. Minor fatty acids were lignoceric acid (24:0), palmitic (16:1ω7), behenic acid (22:0), myristic acid (14:0), α-linolenic (18:3ω3), and arachidic acids (20:0). Other fatty acids were observed in trace amounts. This is in agreement with fatty acid profiles of yeast species as analyzed by other researchers (Amaretti et al., 2012; Botha and Kock, 1993; Rattray, 1988). Fatty acid profiles vary significantly between species, to a lesser degree among strains of the same species, also for the same strain grown under different growth conditions. For example, total lipid content as well as fatty acid profile of several

strains of *R. glutinis* varied considerably between strains, and for the same strain grown under slightly different conditions (Sitepu et al., 2013).

2.3.7 Biodiesel predicted properties based on the fatty acid composition of yeast oil

Although research concerning yeast oil biodiesel properties is not covered sufficiently in the literature, statistical models may provide a useful tool to predict biodiesel properties based on yeast oil fatty acid composition (Pinze et al., 2011). In this sense, table 2.5 summarises the potential values for yeast oil biodiesel cold filter plugging point (CFPP), flash point (FP), cetane number (CN), low calorific value (LCV) and kinematic viscosity (m) based on the previous statistical models.

For comparison purposes, properties from most commonly used vegetable oil-based biodiesel (rapeseed and palm oil biodiesel) have been included. As may be seen from table 2.5, no significant differences in terms of LCV and FP between the oils were found. According to cetane number, that indicates the ignition quality of the fuel, most samples showed a higher value compared to rapeseed oil biodiesel CN, although the value was slightly lower or similar compared to that of palm oil biodiesel. In any case, all samples fulfilled European standard for biodiesel EN 14214. Concerning CFPP and m values, predictive models for yeast oil biodiesel showed large differences among them and compared to traditional biodiesel. With respect to kinematic viscosity predicted values, only a few oils would meet the limits set by EN 14214 (3.5– 5mm2/s) (Leiva-Candia et al., 2014).

The same problem is experienced by the widely used palm oil biodiesel. Eventually, we can conclude that the analysed yeast oil-based biodiesel could be used as fuel for diesel engines only when they are blended with diesel fuel. As most yeast oils show similar fatty acid composition to palm oil, that is directly related to cold weather behaviour, CFPP indicates most of them would not be advisable for cold climates, unless biodiesel is blended with diesel fuel (Leiva-Candia et al., 2014).

TABLE 2.5 - PREDICTED PROPERTIES FOR YEAST OIL BIODIESEL

(continua)

Oil origin	Substrate	Chain length	Unsaturation degree	Low calorific value, LCV (kJ/kg)	Cetane number, CN	Kinematic viscosity, m (mm2/s)	Flash point, FP (°C)	Cold filter plugging point, CFPP (°C)	References
				-	>51	3.5–5.0	>120	-	EN 14214
Rapeseed		17.72	1.30	37,442.46	56.93	4.89	166.88	-3.16	Leiva-Candia et al., 2013
Palm		17.03	0.61	37, 442.56	69.87	5.46	162.16	8.23	Pinzi S et al., 2011
Cryptococcus curvatus TYC-19	Beet molasses	17.93	1.59	37498.36	52.23	4.66	167.41	5.94	Takakuwa N et al., 2010
Rhodotorula mucilaginosa TJY15a	Cassava starch	17.25	0.77	37485.45	66.88	5.35	164.80	5.39	Andre A. et al., 2010
Lipomyces starkeyi AS2. 1560	Cellobiose	17.09	0.59	37,474.44	70.55	5.52	164.36	5.53	Gong ZW. et al., 2014
Cryptococcus curvatus TYC-19	Cheese whey	17.92	1.54	37,510.35	52.99	4.70	167.95	5.53	Takakuwa N et al., 2010
Yarrowia lipolytica NC-I	Chicken product fat	17.07	0.91	37, 344.29	62.71	5.02	155.62	0.52	El Bialy H et al., 2011
Cryptococcus curvatus ATCC 20509	Hydrogen production effluent	17.04	0.70	37,410.75	67.55	5.32	159.89	5.58	Chi ZY. Et al., 2011
Rhodotorula mucilaginosa	Molasses	16.09	0.35	37, 082.19	71.75	5.24	141.12	7.72	Karatay SE. Et al., 2010
Candida curvata NRRL-Y 151	Raw glycerol	17.46	0.72	37,607.47	69.16	5.57	173.55	8.59	Chatzifragkou A, et al., 2011
Candida oleophila ATCC 20177	Raw glycerol	17.44	0.90	37,526.97	64.51	5.27	167.56	3.32	Chatzifragkou A, et al., 2011
Zygosaccharomyces rouxii LFMB 3	Raw glycerol	17.09	0.96	37,332.90	61.75	4.96	154.97	61.75	Chatzifragkou A, et al., 2011
Rhodotorula sp. LFMB 22	Raw glycerol	17.27	0.82	37,478.33	65.76	5.29	164.27	4.22	Chatzifragkou A, et al., 2011
Yarrowia lipolytica LFMB 19	Raw glycerol	17.37	0.93	37,480.87	63.49	5.18	164.41	2.07	Chatzifragkou A, et al., 2011
Pichia membranifaciens	Raw glycerol	17.72	0.97	37, 637.70	64.07	5.35	175.47	3.59	Chatzifragkou A, et al., 2011

TABLE 2.5 - PREDICTED PROPERTIES FOR YEAST OIL BIODIESEL

(conclusão)

Oil origin	Substrate	Chain length	Unsaturation degree	Low calorific value, LCV (kJ/kg)	Cetane number, CN	Kinematic viscosity, m (mm2/s)	Flash point, FP (°C)	Cold filter plugging point, CFPP (°C)	References
Yarrowia lipolytica ACA-YC 5033	Raw glycerol	17.59	0.94	37, 584.68	64.28	5.31	171.62	3.46	Andre A. et al., 2009
Yarrowia lipolytica LFMB 19	Raw glycerol	17.48	1.03	37, 497. 00	61.80	5.11	165.55	0.64	Andre A. et al., 2009
Yarrowia lipolytica LFMB 20	Raw glycerol	17.46	0.92	37, 530.89	64.25	5.26	167.83	3.09	Andre A. et al., 2009
R. toruloides AS21389	Raw glycerol	17.09	0.66	37,449.83	68.75	5.41	162.54	7.08	Xu JY. et al., 2012
R. toruloides AS21389	Raw glycerol	16.89	0.67	37, 349.35	5.23	155.99	5.21		Xu JY. et al., 2012
Lipomyces starkeyi	Olive oil mill wastewater	17.52	0.98	37, 535.47	63.08	5.21	168.15	2.02	Yousuf A. et al., 2010
Lipomyces starkeyi DSM 70295	Sewage sludge	16.43	0.28	37, 274.19	75.45	5.59	152.32	13.29	Angerbauer C, et al., 2008
Cryptococcus albidus ATCC 10672	Volatile fatty acids	17.69	1.40	17, 453.24	54.91	4.74	163.61	4.74	Fei Q et al., 2011

FONTE: Leiva-Candia et al., 2014

2.4 . THE GENUS RHODOTORULA FOR BIODIESEL PRODUCTION

Rhodotorula glutinis is capable of synthesizing numerous valuable compounds with a wide industrial usage. Biomass of this yeast constitutes sources of microbiological oils, and the whole pool of fatty acids is dominated by oleic, linoleic, and palmitic acid. Due to its composition, the lipids may be useful as a source for the production of the so-called third-generation biodiesel. These yeasts are also capable of synthesizing carotenoids such as β-carotene, torulene, and torularhodin. Due to their health-promoting characteristics, carotenoids are commonly used in the cosmetic, pharmaceutical, and food industries. They are also used as additives in fodders for livestock, fish, and crustaceans. A significant characteristic of R. Glutinis is its capability to produce numerous enzymes, in particular, phenylalanine ammonia lyase (PAL). This enzyme is used in the food industry in the production of L-phenylalanine that constitutes the substrate for the synthesis of aspartame—a sweetener commonly used in the food industry (Kot et al., 2016).

Until recently, the yeasts of the genus *Rhodotorula* were primarily considered to be saprophytes that spoil food. In recent times, a large number of studies have been published on the biotechnological uses of these yeasts, which suggest that they may constitute important group of microorganisms that might be of importance in industries in the future, *Rhodotorula glutinis* is considered to be the typical species of this genus.

2.4.1 LIPID BIOSYNTHESIS BY *Rhodotorula glutinis*

In recent years, there has been an increased interest in developing new methods to obtain lipids from these yeasts. One such method includes the production of microbiological lipids, referred to as SCO in the literature (single cell oil) (Beopoulos and Nicaud 2012). In comparison to the production of vegetable and animal fats, this method is independent of climate, season, and geographical position of a country. Production cycle is short, thanks to the rapid growth rate exhibited by the microorganisms (Santos et al. 2013). Microbiological lipids can be used as food additives, diet supplements, and substitutions for precious fats. Microbiological oils can also be used as substrates in the so-called third-generation biodiesel production (Li et al. 2008; Papanikolaou and Aggelis 2011b; Papanikolaou et al. 2001, 2003; Ratledge and Cohen 2008).

The yeast *Rhodotorula glutinis* belongs to the group of oleaginous microorganisms (Table 1), which are defined as those that are capable of producing and accumulating over 20 % of lipids in dry cellular substance (Ratledge and Cohen 2008). Fat is stored in the lipid bodies (Ham and Rhee 1998), whose structure is similar in all oleaginous yeasts. The core consists of backup hydrophobic compounds (such as triacylglycerols, free fatty acids, and sterols), and it is surrounded by a layer of phospholipids bound to proteins (Fickers et al. 2005). In these yeasts, the lipid bodies consist of neutral lipids in the form of triglycerides, and the composition of phospholipids differs from this composition in other celular organelles. This stems from the fact that they primarily consist of phosphatidylcholine (38.6 %) and phosphatidylserine (43 %) (Ham and Rhee 1998).

2.4.2 FATTY ACID PROFILE OF LIPIDS SYNTHESIZED BY *R. glutinis* AND ITS USES

Lipids synthesized by *R. Glutinis* contain primarily palmitic (C16:0), oleic (C18:1), linoleic (C18:2), and linolenic acids (C18:3). The main fatty acid included in the lipids synthesized by *R. Glutinis* is oleic acid, and its percentage in the total pool of fatty acids may exceed above 60 %. The linoleic acid percentage ranges from above 5 to 25 %, and palmitic acid constitutes on average of above 10–30 %. Low percentage of the fat synthesized by these yeasts is characteristic of stearic acid (to 10 %); however, in certain strains, its content may reach up to 25 %.

Profile of fatty acids synthesized by the *R. Glutinis* primarily depends on the yeast strain and composition of the culture medium (Zhang et al. 2011). However, the composition of the lipids can also be adjusted by modifying the molar ratio C/N in the culture medium (Braunwald et al. 2013), temperature of cultivation (Suutari et al. 1990), and by genetic modification of yeast (Shichang et al. 2013). A significant impact on the profile of fatty acids synthesized by the R. Glutinis yeast has also time of cultivation (Mast et al. 2014; Zhang et al. 2011). Zhang et al. (2011) noted that increasing the time of cultivation *R. glutinis* ATCC 15,125 yeast increased content of unsaturated fatty acids, from 46 (0 h) to 63.1 % (233 h). At this time, oleic and linoleic acids contente increased from 26.9 and 8.5 to 43.8 and 12.7 %, respectively. Significant impact on the profile of fatty acids has also the molar ratio of carbon to nitrogen (C/N) in the culture medium. Braunwald et al. (2013) observed that the content of saturated fatty

acids C16:0 and C18:0 in the biomass of *R. glutinis* ATCC 15125 was the lowest after cultivation in the médium with the initial C/N 20. Also, in this case, authors observed the highest concentration of oleic C18:1 (39.9–44.4 %) and linoleic acids C18:2 (31.2–42.3 %). In contrast, the highest content of linolenic acid (6.54–7.35 %) was determined in yeast cells cultivated in media with a high C/N ratio equal 70 and 120, while in the medium with the initial C/N 20 was significantly lower (3.67–3.97 %).

Composition of fatty acids synthesized by *R. glutinis* is also dependent on temperature of cultivation. Changes in the proportions of fatty acids are one of the factors of yeast adaptation to life in environments with different temperatures. At a lower temperature, yeasts synthesize more unsaturated fatty acids, which is associated with changes of the cell membranes (Zlatanov et al. 2010). Suuta et al. (1990) found that the *Rhodosporidium toruloides* VTT-C-132 82 (teleomorph stages of *R. glutinis*) synthesized the largest amount of linoleic acid (approx. 22 %) at 10 °C. The content of this acid after cultivation at 40 °C was only approx. 10 %. Lipids synthesized by *R. glutinis* can be also enriched in linoleic acid through genetic modification. Shichang et al. (2013) used for this purpose implantation of nitrogen ions. The obtained mutant D30 synthesized almost 3-fold more linoleic acid (27 %) compared to the parental strain *R. glutinis* 31,596 (9.93 %), while significantly reduced oleic acid (from 61.8 to 49.3 %) and palmitic acid (from 5.66 to 11.0 %). Due to the participation of individual fatty acids in the lipids synthesized by *R. glutinis*, researchers have indicated the possibility of using these yeasts as a source of substrates for biodiesel production (Dai et al. 2007; Liu et al. 2015; Mast Et al. 2014; Saenge et al. 2011a; Schneider et al. 2013; Xue et al. 2010; Yen and Zhang 2011b; Zhang et al. 2011).

2.4.3 Other potential of *Rhodotorula glutinis*

2.4.3.1 Carotenoid biosynthesis by *Rhodotorula glutinis*

Carotenoids belong to the group of natural pigments found in fruits, vegetables, fish, eggs, and oil (Rao and Rao 2007). Additionally, they are synthesized by certain microbes, including *R. glutinis* yeast (Perrier et al. 1995). They are characterized by yellow, orange, or red coloration. Until now, approximately 750 compounds of this type have been identified (Maoka 2011), out of which 50 compounds exhibit provitamin A activity (Fraser and Bramley 2004). Humans are unable to biosynthesize carotenoids,

and therefore, they must be supplied with diet (Woodside et al. 2015). These compounds are highly soluble in fats but they do not dissolve in water. Carotenoids exhibit health promoting activity toward human body. Thanks to their antioxidant properties, they protect the skin against the UV light. They possess antioxidative effect against free radicals as well as reactive oxygen species.

They strengthen the immune system and accelerate wound healing. Some carotenoids may be protective in eye disease because they constitute vitamin A precursors (Krinsky and Johnson 2005; Rao and Rao 2007). Carotenoids are used in various industrial sectors as componentes of cosmetics (Anunciato and da Rocha Filho 2012) and additives to fodders for livestock (Chatzifotis et al. 2005) and fish (Gouveia et al. 2003). They are also commonly used in food industry as food pigments (Carocho et al. 2015). According to the data published in the report bthe Global Market for Carotenoids in 2014, the world's carotenoid market has achieved a value of 1.5 bn USD, and it is forecast that in 2018, it will increase to 1.8 bn USD (BCC Research 2016) (Kot el a., 2016)

The increasing consumer awareness on the negative effect of synthetic pigments on human health and on the healthy diet trend causes increasing interest in natural pigments (Panesar, 2014). The use of microorganisms as bioreactors for the production of carotenoids can constitute an alternative for chemical synthesis (Del Campo et al. 2007). Microbiological synthesis is a more effective method in comparison to extraction from vegetables or chemical synthesis. The most important advantages of the process include the possibility to decrease its costs by the use of improved strains and inexpensive (often waste) carbon and nitrogen sources in culture media (Buzzini 2000).

2.4.3.2 INDUSTRIAL USE OF CAROTENOIDS PRODUCED BY *R. glutinis*

β-Carotene is the most desired carotenoid type, commonly used as pigment in foods and diet supplements (Carocho et al. 2015; Schierle et al. 2004). Currently, torulene and torularhodin are not commercially used. It is generally known that torulene ($C_{40}H_{54}$) exhibits properties of provitamin A and antioxidative effect (Maldonade et al. 2008). It was determined in an in vitro study that torularhodin ($C_{40}H_{52}O_2$), carboxylated

torulene derivative, has greater capacity to neutralize free radicals compared to β-carotene (Sakaki et al. 2001). Scarce scientific publications have indicated the possibility to use torulene and torularhodin as components of cosmetics and food (Zoz et al. 2015), and as ingredients of drugs (Ungureanu and Ferdes 2012). Toxicity studies conducted on rats demonstrated that β-carotene, torulene, and torularhodin produced by *R. glutinis* DFR-PDY yeasts can be used as safe food additives (Latha and Jeevaratanm, 2012). The capacity of *R. glutinis* to synthesize carotenoids can be also used for medical purposes, for example, dried and powdered *R. glutinis* NCIM 3353 yeasts biomass added to the fodder for rats. It was determined that it exhibited protective effects against the precancerous lesions of the liver induced by N-nitrosodimethylamine (Bhosale et al. 2002). Moreover, torulene and torularhodin inhibit the growth of prostate cancer (Du et al. 2016). Torularhodin can be also used as a neuroprotective agente against H_2O_2-induced oxidative stress, due to its strong antioxidante activity (Wu et. al., 2015).

2.4.3.3 BIOSYNTHESIS OF PHENYLALANINE AMMONIA LYASE BY *R. glutinis*

R. glutinis, depending on the culture conditions, has the capacity to synthesize different types of enzymes that can be used in various industrial sectors. It was determined that the biomass of these yeasts can be source of lipases (Hatzinikolaou et al. 1999; Khayati and Alizadeh 2013; Papaparaskevas et al. 1992), α-L-arabinofuranosidase (EC 3.2.1.55) (Martínez et al. 2006), invertase (EC 3.2.1.26) (Canli et al. 2011; Rubio et al. 2002), pectinases, and tannin acyl hydrolase (EC 3.1.1.20) (Taskin 2013). However, researches have focused primarily on the possibility to obtain phenylalanine ammonia lyase (E.C.4.3.1.5). As a result of the effect of this enzyme, it is possible to obtain L-phenylalanine, which constitutes the substrate for aspartame production (D'Cunha et al. 1996a, 1996b; Zhu et al. 2014).

Phenylalanine ammonia lyase (PAL) catalyzes the nonoxidative process of phenylalanine transformation to trans-cinnamic acid and ammonia (D'Cunha et al. 1996a, 1996b). Under controlled conditions, this reaction may also take place in a reverse direction (Takac et al. 1995). In the food industry, this enzyme is used in the production of L-phenylalanine and para-hydroxycinnamic acid (Cui et al. 2015), and in medicine in phenylketonuria therapy (Longo et al. 2014; Sarkissian and Gámez 2005) and neoplastic cancers in mice (D'Cunha 2005). Furthermore, the activity of PAL is

used to determine the concentration of L-phenylalanine in blood plasma (Watanabe et al. 1992).

2.5 OPTIMIZATION OF PROCESS PARAMETERS TO INCREASE LIPID PRODUCTION

As described in reviews (Sitepu et al. 2014; Ratledge, 2002; Ratledge and Wynn, 2002; Rattray, 1988; Woodbine, 1959), it has been known for decades that yeast lipid content is affected bymany factors including the yeast species, yeast strain, stage of growth, C/N ratio, carbon source and level, aeration rate, temperature, presence of alcohol, nitrogen level, nitrogen source, phosphorus level, thiamine, biotin, pH, and acclimatization to a carbon source. Several studies have applied this knowledge to optimize process parameters to increase lipid and cell mass production when oleaginous yeasts were grown on various carbon sources and waste streams (Angerbauer et al., 2008; Beopoulos et al., 2010; Gong et al., 2012; Thiru et al., 2011; Wiebe et al., 2012; Wu et al., 2010; Zhao et al., 2008; Zhu et al., 2008).

2.5.1 NITROGEN SOURCE AND LEVEL

The effects of nitrogen source and levels on total lipids and on fatty acid profiles has been analyzed with varying results, depending on the yeast species being examined (Evans and Ratledge, 1984). In a study of 17 species of yeasts, Evans and Ratledge (Evans and Ratledge, 1984) observed that lipid content varied little among most species grown in ammonium chloride, asparagine and glutamate; an exception was Rhodosporidium toruloides, which had much higher lipid accumulation when grown on organic nitrogen than inorganic nitrogen. Hansson and Dostalek (Hansson and Dostalek, 1986a, b) observed almost identical growth curves with Cryptococcus albidus grown in several nitrogen sources.

Sitepu et al. (2013) cultured dozens of yeast strains in a defined low nitrogen medium (Medium A) (Sutari et al., 1990) for three days (Culture A), for five days (Culture B), or for a total of five days with a shift from low nitrogen to nitrogen-free medium at three days (Culture C). Total lipid was measured gravimetrically. This work confirmed earlier observations that nitrogen deficiency induces higher lipid

accumulation in oleaginous yeasts, as 35 out of 37 oleaginous yeast species tested had highest overall lipid content in Culture C.

2.5.2 CARBON/NITROGEN RATIO

The C/N ratio in the growth medium has been observed to have a strong effect on lipid production in many yeast species, as summarized in many book chapters and reviews (Lundin, 1950; Ratledge, 1989; Rattray, 1988; Turcotte and Kosaric, 1989; Woodbine, 1959). Most known oleaginous yeasts accumulate more lipid as the C/N ratio rises to a certain point, and lipid accumulation is triggered when nitrogen is depleted from the medium. For example, increasing the molar C/N ratio from 150 to 350 increased the glucose-to-lipid conversion yield by *Rhodotorula glutinis* from 0.25 to 0.40, but increasing the C/N ration further had detrimental effects on yeast cell viability (Beopoulos et al., 2009). *Cryptococcus terricola* is an exception: it has been observed to accumulate lipid during rather than after logarithmic growth, and in the presence of excess nitrogen (Pedersen, 1961, 1962). The presence of more than one carbon source greatly affects lipid productivity: in Y. lipolytica, the optimal molar C/N ratio for best lipid production was 35 if only glucose was used, but 180 if a combination of glucose plus glycerol was used. Kraisintu et al. (2010) measured cell mass and lipid production of one strain of *R. toruloides* under several conditions including varying C/N ratio. They found that the nitrogen source, glucose level and C/N ratio had significant effects. In agreement with previous reports, increasing the C/N ratio decreased cell mass but increased lipid content; overall lipid productivity was increased only slightly at the highest C/N molar ratios of 115 and 140, with a maximum cell lipid content of 62.3% at C/N ratio of 140.

2.5.3 AERATION RATE AND DISOLVED OXYGEN (DO) CONCENTRATION

Aeration rate and dissolved oxygen (DO) concentration It has been known for almost 100 years that thorough aeration promotes lipid accumulation by oleaginous yeasts (Woodbine, 1959). Before the development of modern bioreactors, less successful attempts to increase the dissolved oxygen content of oil-accumulating yeast cultures included growth in shallow pans, and trickling media over suspended fabric belts (Woodbine, 1959). However, increasing aeration is not guaranteed to increase lipid accumulation. Increased lipid production has been observed in *S. cerevisiae* and

C. utilis in some cases under aerobic growth, and in other conditions under anaerobic growth (Rattray et al., 1975). Optimizing overall productivity, not just final cellular oil content, must be the goal of process development. For example, in a study of the effects of aeration rate on both cell mass and lipid accumulation by *Rhodosporidium toruloides* (formerly called *Rhodotorula gracilis*), faster growth rates correlated with lower final lipid content; the lipid production rate was constant at about 0.012 g lipid/g dry cell mass/h (Choi et al., 1982). Turcotte (Turcotte and Kosaric, 1988) observed that in batch cultures of *R. toruloides*, oxygen demand increased for a time shortly after nitrogen depletion from the medium, then dropped. Correlation of oxygen demand with lipid production appears to vary by yeast species, and must be determined empirically.

2.5.4 SUGAR TYPE AND CONCENTRATION

Different yeast species are able to utilize different carbohydrates that comprise lignocellulosic hydrolysates. A yeast strain should be selected that is able to convert the majority of sugars present in a given hydrolysate into the desired lipids. Furthermore, a yeast must be selected that can tolerate the osmolarity of the hydrolysate. For example, yeast strain *R. toruloides* DMKU3-TK16 produced increasing lipid yield in media containing up to 70 g/L glucose, but lipid and cell mass production decreased at higher glucose concentrations (Kraisintu et al., 2010). The yeast strain *Rhodotorula glacialis* DBVPG 4785, on the other hand, continued to increase in lipid production and cell mass up to an optimal glucose concentration of 120 g/L (Amaretti et al., 2010).

Sitepu et al. (2014) compared cell mass (measured by OD600 and cell dryweight) and lipid accumulation (measured gravimetrically (Sitepu et al., 2012) of six oleaginous yeasts grown in 100 mL of Medium A containing HMF (0.15 mg/mL) and furfural (1.0 mg/mL) plus glucose, xylose or both. Candida intermedia UCDFST 11-455 and UCDFST 11-472; *Candida kazuoi* UCDFST 10-874; *Candida sagamina* UCDFST 10-1002; *Candida tenuis* UCDFST 11-461 and *Cryptococcus magnus* UCDFST 10-900 grew to higher cell density on glucose than on xylose, but had higher overall oil productivity on xylose as a sole carbon source than on glucose. (This finding is in contrast to results seen recently by Wiebe et al. (Wiebe et al., 2012) in a study of R. toruloides, in which glucose gave the highest lipid production.) Strain *Cryptococcus*

magnus UCD-FST 10-900 had the highest lipid yield of the six strains tested, on all three media, with close to 60% oil by cell dry weight.

2.5.5 pH

On an industrial scale, control of pH adds to production costs. Use of yeasts that can accumulate oil despite fluctuations in pH or acidification of the media would be preferable. Yeasts in general are acid-tolerant, which has made them useful for low pH food and beverage fermentations such as wine, pickles, olives and kimchi. Acidified YM médium (pH 3.9) is a simple selective media that has been used for many decades to cultivate yeasts out of mixed populations containing both yeasts and bacteria (Miller and Webb, 1954). Acidic conditions are also used industrially to deter growth of many potentially contaminating bacteria, such as conversion of sugarcane bagasse to ethanol. No deleterious effects of low pH on oil accumulation in oleaginous yeasts have been observed (Turcotte and Kosaric, 1989). For example, Kessell et al., (1968) observed no overall change in lipid productivity in *R. toruloides* grown in pH 3.0, 4.5 or 6.0.

2.5.6 TEMPERATURE

Decreased growth temperature from 25 °C to 10 °C resulted in increased total lipid and phospholipid, and increased unsaturation in low-lipid species *C. utilis* and *S. cerevisiae*, oleaginous species *Y. lipolytica*, and psychrophilic species *Candida scottii* (Brown and Rose, 1969; Hunter and Rose, 1972; Kates and Baxter, 1962; Kates and Paradis, 1973; McMurrough and Rose, 1973). However, operating at low temperatures at a production scale could be prohibitively expensive because of the chilling capacity necessary.

2.5.7 OTHER FACTORS

Additional medium components can impact lipid accumulation by yeasts. While the effects of nitrogenous compound concentration and C/N ratio on lipid content have been extensively examined, the effects of phosphorus and sulfur compounds on lipid content has not been studied as extensively, though some effect is suggested (Lundin, 1950, Rattray, Scheibeci, 1975). Limiting phosphorus in a culture of S. cerevisiae

increased the TAG content (RSK). Increasing the sodium chloride concentration to 10% resulted in a significant increase in lipid content of *Candida albicans* (Rattray, Scheibeci, 1975). Use of a young, actively dividing seed culture is also crucial for some yeast species (Lundin, 1950). Effects on lipid productivity of components of lignocellulosic hydrolysates such as furans and organic acids could be elucidated with further analysis.

2.6 UTILIZATION OF SUBSTRATES LOW-COST FOR SINGLE CELL OIL PRODUCTION

There have been numerous reports of conversion of various waste products and hydrolysates to oil using oleaginous yeasts. Conversion of hydrolysates and waste products to oil by oleaginous yeasts have been discussed in many publications and summarized in reviews (Ageitos et al., 2011, Angerbauer et al., 2008, Chi et al., 2011, Galafassi et al., 2012, Li et al., 2008, Meng et al., 2009; Rattray, 1988; Rattray et al., 1975, Rossi et al., 2011). Examples of recent feedstocks used for single cell oil production are listed in a recent review by Huang et al. (2013a), and include raw materials from the food industry (molasses, tomato waste, sweet sorghum extracts, etc.), hydrophilic materials (molasses), and hydrophobic materials (vegetable oils, industrial fats).

Conversion of wastes and plant materials that have been converted to oil using oleaginous yeasts without prior hydrolysis were recently summarized (Leiva-Candia et al., 2014). Because yeasts lack significant cellulolytic activity, lignocellulosic material must be pretreated and enzymatically hydrolyzed to release free sugars prior to conversion to lipids by oleaginous yeasts.

Lignocellulosic materials available for conversion include many waste streams such as agricultural, food processing, forestry, industrial and municipal, as well energy crops (Van Dyk and Pletschke, 2012). The composition of a given feedstock varies by region and season (Food and Agriculture Organization of the United Nations, 2009). Hydrolysates of these materials vary significantly in properties that affect yeast growth including pH, the amount and type of nitrogen and carbon sources, and amounts and types of fermentation inhibitors such as organic acids and furans. It is therefore importante to select and develop oleaginous yeast strains that are able to utilize the

carbon and nitrogen sources, tolerate the inhibitors, and both grow and accumulate oil at the pH of the target feedstock hydrolysate.

2.6.1 HYDROLYSATE OF OIL PALM EMPTY FRUIT BUNCHES (OPEFB) TO YEAST OLEAGINOUS

The lignocellulosic wastes from forestry, agriculture and agrobusiness are abundant and relatively cheap, not used for food and attractive as raw materials because they are a rich source of polysaccharides with potential use for the production of microbial oils. Lignocellulose consists mainly of lignin and two polysaccharides: cellulose and hemicellulose. At present, the palm oil industry (Elaeis guinensis) has considerably increased its production in Brazil with more 127 thousand hectares planted and with a production of 1,393,873.00 tonne of fresh bunches destined for the production of palm oil and palm (Dos Santos And Oyama, 2016), this expansion has produced large volumes of lignocellulosic by products, including 13.5% (w/w) of pressed palm fiber (PPF), 22% w/w of oil empty fruit palm clusters (OPEFB), and about 5.5% (w/w) of palm kernel (PK) approximately (Pua et al., 2013). Among these by products, EFB are the most interesting biomass because they are composed of celluloses (28 - 42.85%, w / w) and hemicelluloses (20.09 -24.12% w / w) (Medina et. al., 2016; Magyar et al., 2016; Rahman et al., 2007; Kim et al., 2012). The integrated use of these lignocellulosic resources depends on the degradation of these polymers, being hemicelluloses important in the general conversion process (Cavalheiro et al., 2005).

OPEFB are rich in hemicelluloses and can be hydrolysed to produce a mixture of sugars that are potential substrates for the conversion of a wide variety of products (ethanol, organic acids and oils). Acid hydrolysis is a simple and rapid method that is used for the hydrolysis of biomass. When agricultural residues or hard woods are used as raw materials, xylose is the most abundant sugar in hydrolysates acids (Carvalheiro et al., 2005).

2.6.2 PRODUCTION AND CURRENT SITUATION OF OIL PALM (*Elaeis guineensis Jacq*).

African oil palm (*Elaeis guineensis Jacq*.) is a tropical crop grown primarily for the production of palm oil. It is the world’s highest yielding and least expensive vegetable oil, making it the preferred cooking oil for millions of people globally and a

source of biodiesel. Palm oil and its derivatives are also common ingredients in many packaged and fast foods, personal care and cosmetic products, and household cleaners. Driven by demand for these products, palm oil production nearly doubled between 2003 and 2013 (FAOSTAT, 2015) and is projected to continue increasing (Corley, 2009). Palm oil is the most important tropical vegetable oil globally when measured in terms of both production and its importance to trade, accounting for one-third of vegetable oil production in 2009 (Potss et al., 2014). The dominance of palm oil may be explained by the yield of the oil palm crop, over four times that of other oil crops, as well as its low price and versatility as an ingredient in many processed goods (Schmidt et al., 2008).

FIGURE 2.7 - TREES OF AFRICAN OIL PALM (*Elaeis guineensis Jacq*.)

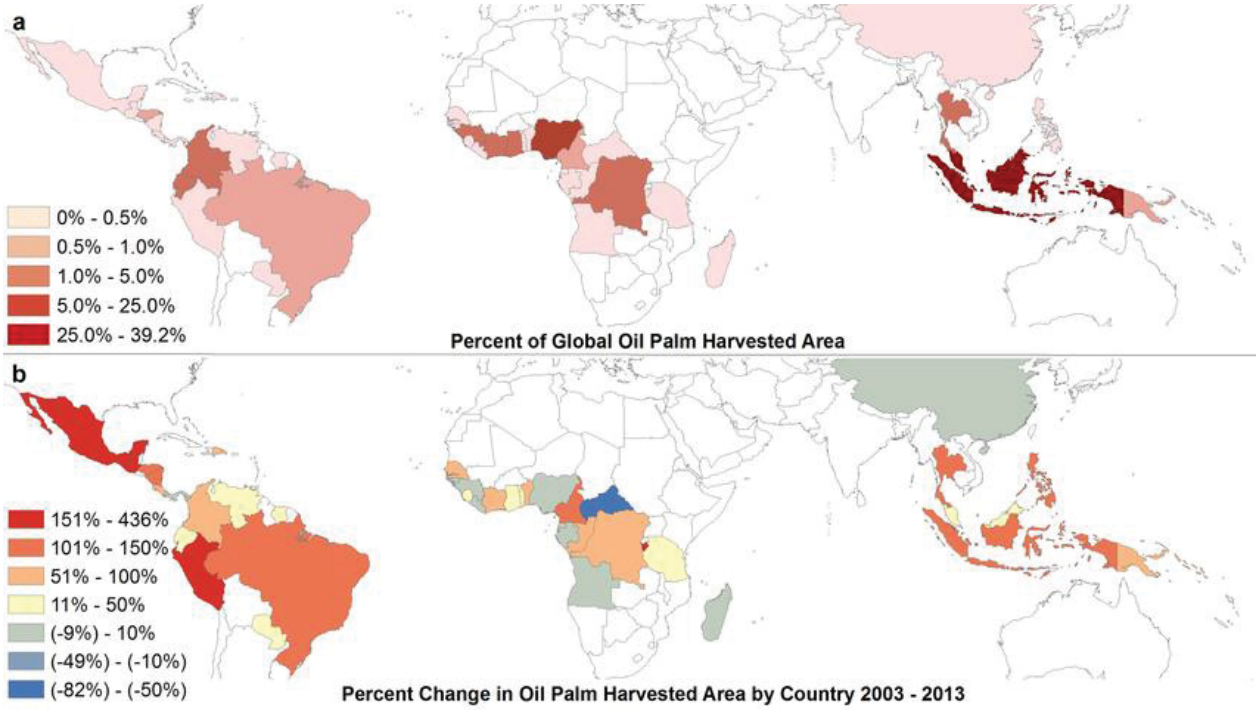
FONTE: Manfred Mielke, USDA Forest Service, Bugwood.org

The growth in demand for palm oil has led to a large expansion of the land used to produce it. Because the oil palm's range is limited to the humid tropics, much of this expansion has come at the expense of species-rich and carbon-rich tropical forests.

Oil palm was responsible for an average of 270,000.00 ha of forest conversion annually from 2000–2011 in major palm oil exporting countries (Henders et al., 2015). One study found that >50% of Indonesian and Malaysian oil palm plantations in 2005 were on land that was forest in 1990 (Koh et al., 2008). Oil palm is currently grown in 43 countries (Figure 8a) so understanding the environmental impacts at a global level may help in understanding differences in development patterns that have led to deforestation. Figure 8b shows the percent growth in oil palm harvested area from 2003–2013. Despite having little plantation area currently, some countries in Latin

America and Africa experienced greater percent growth during this period than did either Indonesia or Malaysia.

FIGURE 2.8 - WORLD PRODUCTION OF PALM OIL. (A) PERCENT OF FAO REPORTED TOTAL GLOBAL OIL PALM HARVESTED AREA IN 2013. (B) PERCENT CHANGES IN FAO REPORTED OIL PALM HARVESTED AREA BY COUNTRY FROM 2003 2013.



FONTE: Vijay et al., 2016.

Specialists estimate that the worldwide production of palm oil will reach about 58 million tons in 2014. Globally, the culture occupies 8% of the lands allocated for the growing of oleaginous. Palm provides almost a third of the global production of vegetable oils. Malaysia and Indonesia are responsible for 85% of the worldwide production. Nigeria, Thailand, Colombia, Ecuador and Papua New Guinea represent, together, 6,6% of the production. The 8,4% that remains is divided among 36 other countries, including Brazil – which is far from being considered a great producer of palm oil (USDA Agriculture Foreign service, 2011)

Brazil produces about 370 thousand tons of palm oil. The biggest part comes from the territory of Pará. However, the national production cannot supply the internal demand that nowadays is, approximately, 500 thousand tons per year, which means that the country imports the product instead of producing it in degraded areas, which would help to improve the balance of emission of greenhouse gases in Amazonian agriculture. Besides, palm is the oleaginous with most productive economic value that

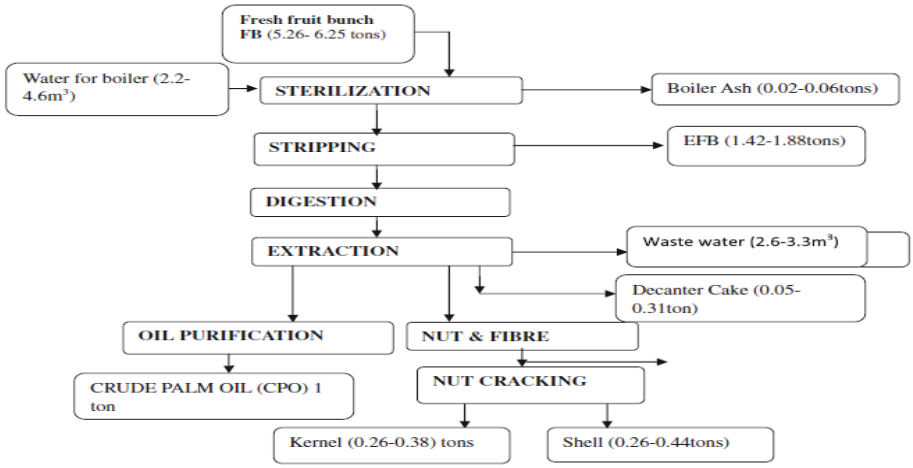
is known of. Its average productive is of 4 tons of oil per hectare/tear, ten times more than soy oil, for instance. In other words, it is more competitive in the global market than similar products (www.abrapalma.org).

2.6.3 LIGNOCELLULOSIC RESIDUES FROM OIL PALM EMPTY FRUIT BUNCHES (OPEFB)

The oil palm has augmented negative impacts to biodiversity, environmental menace and has raised social concerns (Obidzinski et al., 2012). The rise in plantations has significant direct and indirect threats to the environment, and most direct outcome in the country is clearance and felling of natural vegetation and its replacement by oil palm monoculture (Motel et al. 2009).

The plentiful lignocellulosic residues produced from oil palm industries are oil palm fronds (OPF), oil palm trunks (OPT) and empty fruit bunches (EFB). Palm oil industry in Malaysia produced about 95.3 million tons of dry lignocellulosic biomass in 2009 (Basiron and Simeh 2005; Wan Rosli and Law 2011), as the economic life of oil palm is only 25 years after that the trunks are chopped and make residual wood debris. From the oil mills during palm oil generation process, massive amount of waste like the empty fruit bunch (EFB) and oil palm fronds (OPF) is generated. Approximately 1 tonne of crude palm oil (CPO) is produced from 5.8 tonnes of fresh fruit bunch (FFB) (Pleanjai et al. 2004; Singh et al. 2011) (FIGURE. 2.9).

FIGURE 2.9 - STAGES IN PRODUCTION OF PALM OIL, TYPE AND QUANTITY OF WASTE PRODUCED



FONTE: Singh et al., 2013.

After processing the oil, the palm oil industry generates huge quantity of waste (Figure 7). Fiber, shell, decanter cake and empty fruit bunch (EFB) account for 30, 6, 3 and 28.5 % of the FFB. In 2004, nearly 26.7 million tonnes of solid biomass and an average of 30 million tonnes of POME were generated from 381 palm oil mills in Malaysia (Yacob 2008). During processing in the palm oil mill, more than 70 % (by weight) of the processed fresh fruit bunch (FFB) are residual as oil palm waste (Prasertsan and Prasertsan 1996). Tackling with such a huge quantity of waste from palm oil mill is a gigantic task, as if not dealt properly may lead to environment degradation as they are rich in organic substances (Singh et al. 2010 a, 2011b).

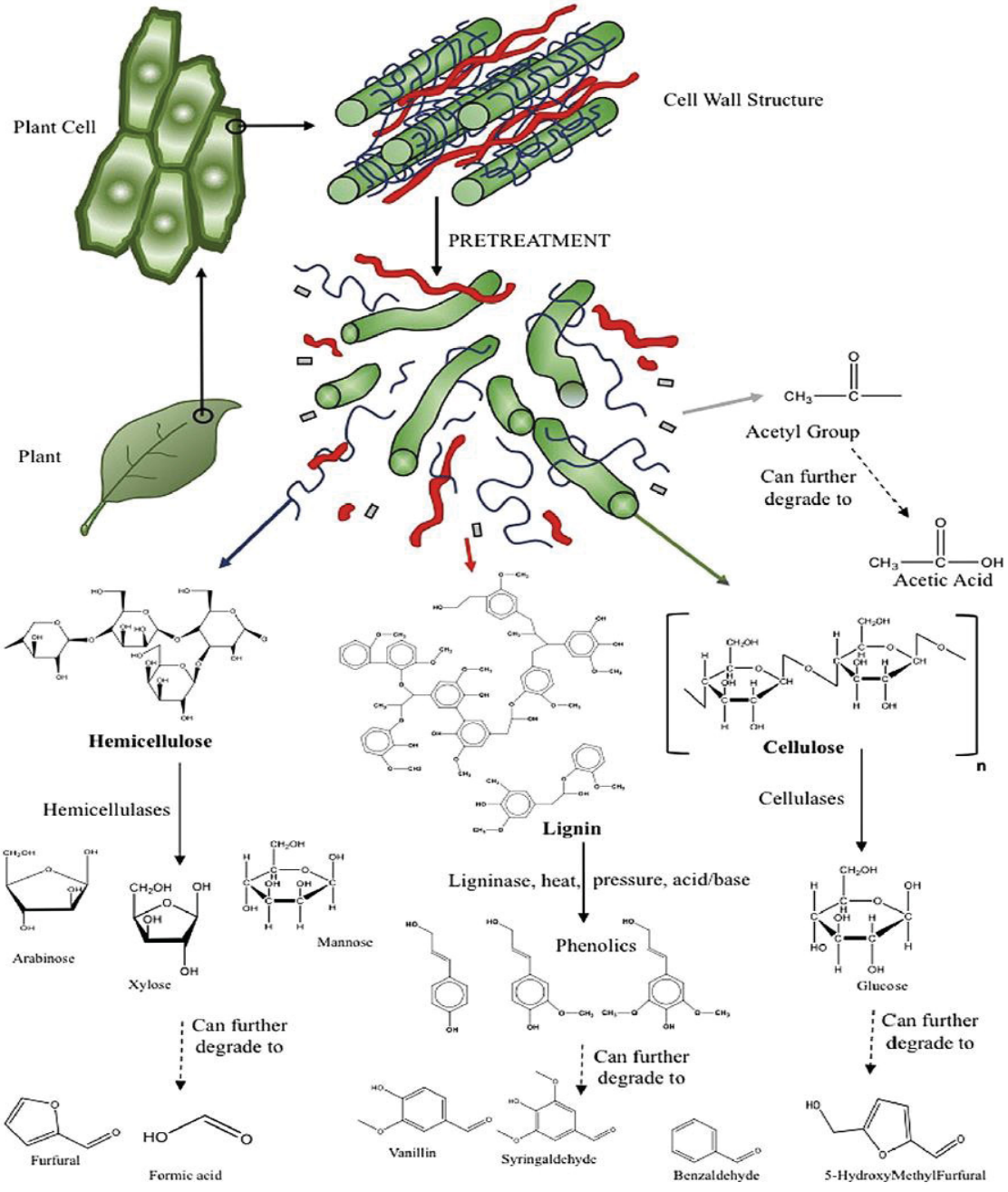
2.6.4 CONVERSION OF LIGNOCELLULOSIC HYDROLYSATES TO YEAST OIL

Oil palm empty fruit bunches (OPEFB) is a lignocellulosic residue of palm oil mill, as discussed above. OPEFB constitutes environmental problem and has low economic value; it is conventionally disposed in land fills, burned openly or used in composting for fertilizer. OPEFB primarily contains 82.4% hollocellulose and 17.6% lignin (Law et al., 2007). Considering the high carbohydrate content of OPEFB, it can be a cheap feedstock for microbial oil and lignocellulosic derivatives such as glucose, xylose, mannose and animal feed (Piarpuzán et al., 2011).

Lignocellulose is composed of several classes of polymers including cellulose, hemicellulose, lignin and pectin (FIGURE 2.10). The relative amounts and types of these polymers varies in different plants (Van Dyk and Pletschke, 2012).

Pretreatment is necessary to open up and separate these polymers, degrading the crystalline structure and making each of the polymers more accessible for enzymatic hydrolysis. A number of biological, physical, chemical, and physico-chemical pretreatment protocols have been developed that are appropriate for different types of plant materials (Alvira et al., 2010, Mosier et al., 2005; Wyman, 1999; Wyman et al., 2005). Because these pretreatment protocols generate different product profiles, a pretreatment regimen must be selected that will generate products that are compatible with the properties of the target yeast strain, or viceversa.

FIGURE 2.10 - LIGNOCELLULOSE STRUCTURE, PRETREATMENT AND HYDROLYSIS PRODUCTS. PRETREATMENT AND HYDROLYSIS OF CELLULOSE, HEMICELLULOSE AND LIGNIN PRODUCE A HYDROLYSATE OF COMPLEX COMPOSITION. SOME OF THE COMMONLY PRODUCED COMPOUNDS ARE SHOWN INCLUDING CARBOHYDRATES, PHENOLIC COMPOUNDS, FURANS, AND ORGANIC ACIDS



FONTE: Sitepu et al. 2014.

After pretreatment, the lignocellulosic material is hydrolyzed to reléase oligo- and monosaccharides. In nature, enzymes of degraders such as bacteria and fungi performthese functions. In industrial applications, these enzymes are harnessed in the form of commercially developed cellulase cocktails, comprised of cellulases,

hemicellulases, pectinases and ligninases. The primary products of enzymatic hydrolysis are illustrated in Figure 2.10.

Cellulose is the primary structural component, made of polymers of glucose joined by β-1,4 linkages, linked by hydrogen bonds to formthe crystalline structure. The cellulose component is surrounded by a matrix of hemicellulose, pectin and lignin. After pretreatment, which separates the components, hydrolysis of cellulose by cellobiohydrolase, endoglucanase, and β-glucosidase releases glucose, cellobiose, and other glucose oligomers. Hemicellulose is comprised of various polymers that vary depending on the species of plant, and can include xylan (the most abundant), galactan, mannan and arabinan. Hydrolysis of hemicellulose by hemicellulases such as endo-xylanase, endo-mannanase, α- glucuronidase, α-galactosidase, and others releases monomers and oligomers that vary depending on the composition of the hemicellulose of the plant species and the activity of the hydrolytic enzyme. These can include D-xylose, D- or L-arabinose, mannose, galactose, and glucose (Beg et al., 2001, Gírio et al., 2010).

Pectin is also a highly diverse class of polysaccharides. It retains water, and is present in higher concentrations in certain high moisture plant materials such as fruits. They are digested by pectinases such as galacturonase, releasingmany sugars such as galacturonic acid (the primary component), rhamnose, arabinose, galactose, and others (Van Dyk and Pletschke, 2012; Willats et al., 2006).

Lignin is a heterogenous, cross-linked, hydrophobic polymer of phenoli monomers. The relative abundance of monomers varies with the type of plant. It is viewed as an obstacle to enzymatic hydrolysis of celulose and hemicellulose (Dijkerman et al., 1997). It is often removed from the pretreated material rather than hydrolyzed, as degradation generates breakdown products that can be toxic to degradative enzymes and fermentative microbes, and the lignin itself could instead be recovered to be burned in boilers to generate energy (Romaní et al., 2010; Van Dyk and Pletschke, 2012). Breakdown or removal of lignin can accomplished by mechanical, thermal, chemical or enzymatic processes such as acid hydrolysis, steam treatment or alkaline treatment (Van Dyk and Pletschke, 2012). Degradation of lignin can release a broad variety of polyphenols and low molecular weight phenolics, such as syringaldehyde, 4-hydroxybenzoic acid, vanillic acid, and vanillin, and other

compounds that can be inhibitory to enzymes or fermentativemicrobes (Palmqvist and Hahn-Hagerdal, 2000; Parawira and Tekere, 2011; Van Dyk and Pletschke, 2012). These compounds also have the potential to be recovered as high-value chemical intermediates.

2.6.5 PRETREATMENT METHODS

In a biorefinery based on lignocellulosic materials, which has sugars as intermediates, it is necessary to break down the feedstock’ s structure, and then to get sugars from celulose and hemicellulose. For this reason, we need a pretreatment system before the conversion step; the pretreatment goal is to prepare the feedstock in order to improve the sugars conversion. TABLE 2.6, is based on information reported by Hu et al. (2008) and Kumar et al. (2009), and it shows a classification and some characteristics of pretreatment methods.

Biological and physical pretreatments based on irradiation and electric means are not ready to be used on an industrial scale, at least in the short term. Comminution types are impractical to be implemented in large scales because oftheir high energy requirements. The chemical oxidant option seems too expensive and produces low yields. Carbon dioxide explosion is used simultaneously with the organosolvent type of pretreatment (Pasquini et al., 2005) or as a second step after dilute acid pretreatment (Zheng et al., 1995, 1998). This effect could be evaluated later as an improvement of organosolvent or dilute acid pretreatments.

Pretreatment methods such as physics based on liquid hot water or steam explosion and chemical pretreatment methods based on dilute acid, alkaline extraction, ammonia fiber blast and organo-solvents seem to have the best potential for implementation within a microbial oil production process.

TABLE 2.6. TYPES OF PRETREATMENT METHODS.

Pretreatment	Energy source	Effect	Advantages and disadvantages
Biological	Microbe (fungi, actinomycetes)	Remove lignin	Low energy consumption, very slow rate of hydrolysis, waste materials by degradation
Physical	Comminution (ball, colloid, hammer, compression)	Decrease particle size and cellulose crystallinity	High power consumption; usually higher than inherent biomass energy.
	Irradiation (electron beam, gamma-ray, microwave)	Increase surface area and pore sizes. Soften and partially deploymerize lignin	Few research in this field.
	Electric (pulsed electrical field)	Disrupts plant cells Ambient conditions, simple equipment.	Process needs more research.
	Hydrothermolysis (liquid hot water)	Partial hydrolysis of hemicelluloses .	Lower pressure and lower destruction of xylan than steam explosion
	Steam explosion (hig pressure Steam)		Simple process, destruction of a portion of xylan, incomplete disruption of the lignin.
Chemical	Acid hydrolysis (carbonic, hydrochloric, hydrofluoric, nitric, phosphoric, sulfuric)Alkaline extraction (calcium, sodium and ammonia hydroxide)	Decrease cellulose crystallinity, partial or complete hydrolysis of hemicelluloses, delignification	Short residence times, high hemicelluloses conversions. Equipment corrosion. At low temperature and low pressure long residence time is required. Part of irrecoverable salts formed.
	Carbon dioxide explosion		Do not produce inhibitors; do not modify lignin or hemicelluloses.
	Ammonia fiber explosión (AFEX)		Do not produce inhibitors; it is not effective with high lignin contente biomass; high pressure.
	Oxidant (ozone, wet oxidation)		Expensive or only for low lignina content.
	Organosolvent (ethanol–water, benzene–water, ethylene glycol, butanol–water)		High delignification, solvents recycle, some solvents are explosive and inflammable

FONTE: Conde-Mejía et al., 2012

2.6.5.1 DESCRIPTION OF PRETREATMENT METHODS

2.6.5.1.1 LIQUID HOT WATER AND STEAM EXPLOSION

These methods use water without any other chemical. In steam explosion (SE), biomass is treated with saturated steam at high pressure, after which the pressure is suddenly decreased (Kumar et al., 2009). The liquid hot water (LHW) process is similar, but in this case the water is fed as saturated liquid. These methods have some differences. The temperature and pressure for LHW are usually lower than for SE, the biomass loads are larger for SE than for LHW, and because of the different solids concentrations a major inhibition could be observed in SE pretreatment (Garrote et al., 1999). The main component of the operating cost for these methods is the energy required to feed the water either as saturated liquid or saturated steam.

2.6.5.1.2 DILUTE ACID HYDROLYSIS

The acid addition increases hemicellulose solubilization rate in comparison with the LHW or SE methods, so that the degradation effect can be minimized (Lloyd and Wyman, 2005). The most widely studied method based on acid hydrolysis is the dilute sulfuric acid hydrolysis (DA); there are some research works with optimal conditions reported (Aguilar et al., 2002; Pettersson et al., 2003; Chandel et al., 2007; De Avila-Rodrigues and Guirardello, 2008). In order to avoid corrosión problems and the need for an acid recovery system, the use of very low acid concentrations has been proposed to improve the economic feasibility of this pretreatment method (Lloyd and Wyman, 2005). The main processing costs are due to the energy consumption and the acid cost.

2.6.5.1.3 ALKALINE EXTRACTION

Alkaline pretreatment breaks the bonds between lignin and carbohydrates and disrupts the lignin structure; this effect depends on the lignin content of the materials. When low temperatures (50–65 ◦C) are used, residence times can be of several days or even weeks, while using high temperaturas (85–135 ◦C) reduces the residence times to the order of hours (Chang et al., 1998). There are experimental works that

have been reported using sodium hydroxide and calcium hydroxide (LIME) solutions as pretreatment media (Targonski, 1985; Kim and Holtzapple, 2005; Park et al., 2010), which improve the yield in the next steps. Lime is less expensive than sodium hydroxide, and it has been proposed that lime can be recovered by precipitation as CaCO3 using carbon dioxide (Chang et al., 1997, 1998, 2001). If the high temperature lime method is used as pretreatment system, the energy consumption will be na important factor in the economic analysis of this option. The lime recovery cost, or the lime cost, will be another importante economic factor for this process.

2.6.5.1.4 AMMONIA FIBER EXPLOSION

In the ammonia fiber explosion method (AFEX), the lignocellulosic materials are exposed to concentrated liquid ammonia at high pressure, and then the pressure is suddenly reduced (Kumar et al., 2009). Optimal conditions such as ammonia loading, moisture content, temperature and residence time have been reported (Mes-Hartree et al., 1988; Teymouri et al., 2004; Alizadeh et al., 2005). The operating cost of this method depends on the energy consumption and ammonia price. An efficient ammonia recovery process is an importante factor to make this pretreatment method economically feasible.

2.6.5.1.5. ORGANOSOLVENT

This method involves simultaneous prehydrolysis and delignification. A solvent mixture and an acid catalyst are used to break the internal lignin and hemicellulose bonds (Hu et al., 2008). The solvents commonly used are mixtures of water with methanol, ethanol, acetone or glycols, although those solvents must be recovered because they act as inhibitors in the reactions steps. After the biomass is treated with the solvent, two phases are formed; one is called black liquor, which contains the main part of solvent with dissolved lignin and the liquid products (Pan et al., 2005, 2006), and the other one is a dense phase, which contains the main part of remaining solids. In this case, the principal costs are the energy consumption and the energy required to recover the solvent. TABLE 2.7 summarizes the six specific methods selected in this work for their analysis along with the main componentes for the operating costs; the energy consumption is a common factor for all methods (Conde-Mejía et al, 2012).

TABLE 2.7 PRINCIPAL OPERATING COST FOR PRETREATMENT METHODS.

Pretreatment	Principal operating cost
Steam explosion (SE)	Energy consumption
Liquid hot water (LHW)	Energy consumption
Dilute sulfuric acid hydrolysis (DA)	Energy consumption and acid cost
Ammonia fiber explosion (AFEX)	Energy consumption and ammonia cost
Alkali extraction with Ca(OH)2 (LIME)	Energy consumption and lime recovery or lime cost
Organosolvent using ethanol–water mixture as solvent (OS)	Energy consumption and solvent recovery cost

FONTE: Conde-Mejía et al, 2012

2.6.6 INHIBITORY COMPOUNDS

Pretreated, hydrolyzed lignocellulosic biomass contains toxic compounds that may inhibit yeast growth and thus overall lipid productivity. Growth inhibitors include breakdown products of hemicellulose, such as acetic acid; products of the partial degradation of lignin, such as phenolic compounds; and furan derivatives such as furfural and HMF resulting from the degradation of pentoses and hexoses (figure 2.10). Selection of an oleaginous yeast strain suitable for conversion of a given hydrolysate should take into consideration tolerance of the inhibitors present in that hydrolysate. Themost notorious inhibitors of yeast productivity include: 5-(hydroxymethyl) furfural (HMF), furfural, acetic acid, formic acid, levulinic acid, vanillin, and p-benzaldehyde (Palmqvist and Hahn-Hagerdal, 2000; Parawira and Tekere, 2011; Zha et al., 2012; Zhang et al., 2010).

The effects of inhibitors on yeast oil production has been examined (Chen et al., 2009b, Hu et al., 2009). For example, removal of inhibitors from rice straw hydrolysate by overliming followed by adsorption on Amberlite XAD-4 greatly improved lipid yield of oleaginous yeast *T. fermentans* from 1.7 to 11.5 g/L (Huang et al., 2009a). Activated charcoal was demonstrated to be an effective, less costly method for detoxification (Huang et al., 2013b).

Zhang et al. (Zhang et al., 2010) identified a fungal strain, Amorphotheca resinae ZN1, that could be used to detoxify pretreated lignocellulose, resulting in improved lipid production by oleaginous yeast (Huang et al., 2011b). Rather than

removing inhibitors, use of an oleaginous yeast strain that is naturally resistant to relevant inhibitors would be a more costeffective strategy. In a comparison of 10 oleaginous yeast strains belonging to 5 oleaginous species, cell mass accumulation and lipid production were found to be inhibitor- and yeast strain-specific (Chen et al., 2009b, Hu et al., 2009). Acetic acid, formic acid, furfural and vanillin most strongly affected lipid production in these yeasts. One strain of T. cutaneum was shown to be particularly resistant to the inhibitors studied. In another study, Galafassi et al. (Galafassi et al., 2012) demonstrated that a strain of *R. graminis* could be cultivated on undetoxified corn stover hydrolysate, and produce 0.21 g lipid/L/h and reach 34% oil w/w. Selection of an oleaginous yeast that is naturally resistant to the inhibitors, or that can inactivate the inhibitors, would reduce production costs significantly. Characterization of a broad selection of yeast strains would enable selection of yeasts with desired combinations of properties. Sitepu et al. (Sitepu et al., 2014b) compared the inhibitor tolerance of a broad range of oleaginous yeast species. All 48 oleaginous yeast strains tested grew well in the presence of 0.5 g/L 5-hydroxymethyl-furfural (HMF), but only seven could growwell in 1 g/L furfural, and twelve in the presence of 2.5 g/L acetic acid. This finding confirmed that furfural is a strong inhibitor of yeast growth, which is in agreementwith Qi et al. (2013).

2.6.7 STRATEGIES TO COUNTERACT INHIBITION PROBLEMS

Several alternative measures can be taken to avoid problems caused by inhibitors. The concentrations of inhibitors and sugars in hydrolysates depend on the feedstock as well as on the conditions during pretreatment and hydrolysis (Galbe et al., 2007; Larson et al., 1999). Therefore, one possibility is to select less recalcitrant feedstocks and to utilize mild pretreatment conditions. However, it is desirable to utilize different varieties of lignocellulose if production of commodities from renewables should make a major impact on the market for fuels, chemicals, and materials. Furthermore, production of bulk chemicals is yield dependent, which implies that it is not reasonable to accept a poor sugar yield, and consequently a poor overall product yield, due to the use of insuficiente pretreatment conditions. It is also possible to design the fermentation process to avoid problems with inhibition, for example by using simultaneous saccharification and fermentation (SSF) to avoid inhibition of cellulolytic enzymes by sugars, or by using fed-batch or continuous cultivation rather than batch

processes (Olofsson et al., 2008). High yield and productivity, high product titer, and possibilities to recirculate process water are, however, important aspects of the chosen design. Ethanol production from diluted hydrolysates with low sugar content is associated with a high operating cost due to a more expensive distillation process (Lin and Tanaka et al., 2006). There is a variety of different chemical, biological and physical methods that can be used to detoxify slurries and hydrolysates (Larsson et al., 1999; Pienkos and Zhang et al., 2009, Parawira and Tekere et al., 2011). Approaches that have been studied include overliming and treatments with other chemicals, liquid-liquid extraction, liquid–solid extraction, heating and evaporation, and treatments with microbial and enzymatic biocatalysts (TABLE 2.8).

TABLE 2.8. TECHNIQUES FOR DETOXIFICATION OF LIGNOCELLULOSE HYDROLYSATES AND SLURRIES.

Technique	Procedure	Example[a]
Chemical additives	Alkali [such as $Ca(OH)_2$, NaOH, NH_4OH	Alriksson et al., 2005; Alriksson et al., 2006.
	Reducing agents [such as dithionite, dithiothreitol, sulfite	Alriksson et al., 2011
Enzymatic treatment	Laccase	Jönsson et al., 1998;
	Peroxidase	Jönsson et al., 1998
Heating and vaporization	Evaporation	Larsson et al., 1999
	Heat treatment	Ranatunga et al., 2000
Liquid-liquid extraction	Ethyl acetate	Cantarella et al., 2004; Fenske et al.,1998
	Supercritical fluid extraction [such as supercritical CO_2	Persson et al., 2002
	Trialkylamine	Zhu et al., 2011
Liquid–solid extraction	Activated carbon	Parajó et al., 1997
	Ion exchange	Nilvebrant et al., 2001; Sárvári et al., 2004
	Lignin	Björklund et al., 2002
Microbial treatment	Coniochaeta ligniaria	Lopez et al, 2004; Nichols et al., 2008
	Trichoderma reesei	Larsson et al., 1999; Palmqvist et al., 1997
	Ureibacillus thermosphaericus	Okuda et al., 2008

FONTE: Jönsson et al., 2013.
NOTA: [a]The table includes one or two examples of each procedure. Dilution, washing of solid fractions, and techniques based on the fermenting microbe are not included.

Comparisons of different methods for detoxification, or conditioning, indicate that they differ significantly with respect to effects on hydrolysate chemistry and fermentability (Larsson et al., 1999; Cantarella et al., 2004). A common objection against detoxification is based on the assumption that it would require a separate process step. There are number of strategies that concern the fermenting microorganism. The use of large inocula decreases inhibition problems (Chung and

Lee, 1985; Pienkos and Zhang 2009; Cantarella et al., 2004). However, the use of large inocula is considered to be a less attractive solution in an industrial context (Wingren et al., 2003). Using a large inoculum would be a possibility if the microorganism can be recirculated and reused at a reasonable cost. However, if the used fermentation broth contains a lot of solids, the separation of the microorganism could become a tedious task. This is the case in simultaneous saccharification and fermentation (SSF) processes, and as a consequence the use of fresh inocula is considered instead of recycling the microorganism (Wingren et al., 2005). Other possibilities that target the microorganism include selection of microbial species and strains that exhibit resistance to inhibitors. Adaptation of the microorganism to na inhibiting environment, possibly after inducing variation by mutagenesis, serves as an alternative option.

Furthermore, genetic engineering can be employed to obtain transformed hyperresistant microbes. *S. cerevisiae* has been engineered for increased resistance to fermentation inhibitors by overexpression of enzymes conferring improved resistance to phenolics (Larsson et al., 2001a, 2001b), furan aldehydes (Petersson et al., 2006; Gorsich et al., 2006), and aliphatic acids (Hasunuma et al., 2011a, 2011b). Furthermore, overexpression of a transcription factor, Yap1 [95], and of multidrug-resistance proteins (Alriksson et al., 2010) has also generated hyperresistant *S. cerevisiae* transformants.

In some of these cases, hyperresistance to lignocellulose hydrolysates has also been demonstrated (Larsson et al., 2001a; Alriksson et al., 2010). Most of the studies on inhibition have had focus on the fermenting microorganism, while strategies that decrease inhibition of enzymes so far have received relatively Little attention. Since most enzymatic hydrolysis processes involve mixtures of a pretreatment liquid and a solid cellulosic material, there are good reasons to take enzyme inhibition into account.

2.6.8 SUGAR CANE MOLASSES TO YEAST OIL

Molasses can be produced from citrus, wood sugar, sugar beet and sugarcane. Here will be described the different types of molasses that can be produced from clarifying, concentrating and/or extracting sucrose from sugarcane juice in a raw sugar factory and from refining raw sugar in a sugar refinery, as well as their primary use in animal feeds. Sugarcane processing is focussed on the production of cane sugar (sucrose) from sugarcane. Other products of the processing include bagasse,

molasses, and filtercake. Bagasse, the residual woody fiber of the cane, is used for several purposes: fuel for the boilers and lime kilns, production of numerous paper and paperboard products and reconstituted panelboard, agricultural mulch, and as a raw material for production of chemicals. Bagasse and bagasse residue are primarily used as a fuel source for the boilers in the generation of process steam. Thus, bagasse is a renewable resource. Dried filtercake is used as an animal feed supplement, fertilizer, and source of sugarcane wax. Molasses is produced in two forms: inedible for humans (blackstrap) or as an edible syrup. Blackstrap molasses is used primarily as an animal feed additive but also is used to produce ethanol, compressed yeast, citric acid, and rum. Edible molasses syrups are often blends with maple syrup, invert sugars, or corn syrup.

Final molasses is composed of organic and inorganic matter and water. About 52% of blackstrap molasses is total sugars, about 10% or more is inorganic salts or ash, 10-20% water, and the balance is organic non-sugar matter. Potassium and sodium salts are considered as molasses-forming. The analysis of blackstrap, and particularly of sugars in it, may vary considerably depending on the variety of sugar cane, soil, climate, period of the crop, efficiency of the factory operation, system of sugar boiling, type and capacity of crystallizers etc. Blackstrap molasses is a by-product of sugar production, and is sold considerably cheaper than raw sugar. For this reason, it is in the interest of a sugar factory to produce a minimum amount of blackstrap containing a minimum amount of crystallizable sugar (Baikow, 2013)

For a long time, fermentation was usually carried out on varying sugars. Thus, low-cost materials that contain various sugars could be used for microbial oil production. Molasses, from sugarcane or beet, is an industrial by-product of sugar manufacturing. Generally, molasses contains fructose, sucrose, and glucose; therefore, this sugar was considered an ideal raw material for cheap medium culture formulations and was widely used in the fermentation industry for ethanol (Doelle and Doelle, 1990), hydrogen (Tanisho and Ishiwata, 1995), lactic acid (Wee et al., 2004), and levan (Han and Watson, 1992). Sugarcane (Zhu et al., 2008) and beet molasses (Bednarski et al., 1986) have been used for SCO production. More recently, *Cunninghamella echinulata* showed great potential in the decolorization-detoxification of waste molasses and in efficiently using molasses for SCO production (Chatzifragkou

et al., 2010). Although oleaginous microorganisms can grow well on molasses medium due to its high sugar content, the high nitrogen content of this sugar prevents its lipid accumulation (Zhu et al., 2008).

3. CHAPTER I: SCREENING OF NEW OLEAGINOUS YEASTS WITH LIPID PRODUCTIVITY FOR BIODIESEL PRODUCTION

ABSTRACT

The lipid-accumulating ability of 140 yeast strains isolated from of different environments, Parana State, Brazil was evaluated. Screening revealed that 7 strains were identified as potential lipid producers were cultivated in a medium containing 3 % glucose. It was found that PPGEBB115 accumulated the highest lipid content, up to 32.06%. They were tentatively identified as Rhodotorula glutinis PPGEBB115 with lipid concentration of 1.79 g/L. High lipid productivity is the most important characteristic of oleaginous yeasts. Among the strains tested here Rhodotorula glutinis, had the best lipid productivity, 0.456 g/L/day. In addition, 4 isolated yeasts were evaluated on glucose, xylose, glycerol, molasses sugar cane and hydrolysate of oil palm empty fruit bunches (OPEFB), Rhodotorula glutinis PPGEBB115 presented better productivity (0.48 g/L/day) using sugar cane molasses and Rhodotorula mucilaginosa PPGEBB116 presented better productivity of 0.24 g/L/day of hydrolysate of OPEFB, lipid content of 33.04 % and 17.80 %, respectively. The results show that isolated yeasts could be promising candidates for the production of biodiesel due to the composition of the fatty acids present in the oil, such as oleic acid, linoleic acid, palmitic acid and stearic acid.

3.1 INTRODUCTION

As world population continues to grow, there is an ever-increasing demand on energy and material resources. Therefore, to ensure long-term sustainability, suitable alternative production methods for oil as feedstock for several industrial applications must be developed (Schulze et al., 2014). Biodiesel and bioethanol derived from plant oil for example, have been used since time years, but the disadvantage is the competition with the need to produce feed and food (Ratledge 1993). Therefore, oleaginous microorganisms represent an alternative production system for sustainable lipid production as they share the special feature to produce more than 20% lipid per dry biomass as carbon storage reserves with similar fatty acid compositions as plant oils, whithout competition with food crops.

Lipids are produced by all microorganisms usually in the range of 6 to 8% per dry biomass principally as components for the cell membrane. However, oleaginous microorganisms, including yeasts, bacteria, filamentous fungi and microalgae, convert a carbon source when it is available in excess into Intracellular Triacylglycerols (TAG) as soon as a nitrogen limitation occurs (Ratledge, 2002). These lipids are also called Single Cell Oils (SCO) and are stored as lipid droplets within cells.

The advantage of oleaginous yeast is their ability to produce lipids from non-utilized biomass, including lignocellulosic biomass (Gong et al., 2012; Liang et al., 2012). It is known that many oleaginous yeasts such as Rhodosporidium toruloides, Cryptococcus curvatus, Lipomyces starkeyi and Yarrowia lipolytica accumulate lipids to more than 20% of the dry yeast cells (Feofilova *et al.,* 2010; Papanikolaou and Aggelis, 2010; Thiru *et al*., 2011; Wild *et al*., 2010; Wu *et al*., 2011) and that several oleaginous yeasts, including R. toruloides and L. starkeyi, can assimilate xylose as carbon source (Gong *et al.,* 2012; Oguri *et al.,* 2012; Zhao *et al.,* 2012). One of the difficulties in the adaptation of biolipid production by oleaginous yeasts may be due to low productivity (Feofilova *et al*., 2010). In general, the growth of oleaginous yeasts is much slower, compared with that of ascomycetes such as Saccharomyces cerevisiae, besides biolipid production by oleaginous yeasts requires a prolonged period to produce maximum yields (Ageitos *et al.*, 2011). To evaluate the lipid-accumulating ability of oleaginous microorganisms, lipid content (% of dry cell weight) is the most commonly used parameter (Meng *et al.,* 2009; Song *et al*., 2013).

Currently, there has been an increasing interest in utilizing low cost substrates such as residues of agroindustrial or lignocellulosics as carbon source. Among waste materials, lignocellulose is of great importance because it is produced in enormous quantities annually, as a result of land cultivation. Sugars from the hydrolysis of lignocellulosic biomass could be an excellent carbon source for the production of microbial lipid. Chen et al. (2009) screened several oleaginous organisms for their ability to grow and convert lignocellulosic sugars to biomass and triglycerides in the presence of inhibitor compounds generated during the de-polymerization of lignocellulosic sugars.

The hydrolysate of lignocellulosic materials mainly consists of fermentable sugars such as xylose, glucose and arabinose from the hemicellulosic estructure. Accompanying these monosaccharides in the hydrolysis of lignocellulosic materials, soluble materials such as lignin, acetic acid and furfural also can be produced, which can inhibit both growth and sugar utilization of microorganisms during fermentation (Cheng et al., 2008).

Xylose and glucose are the main products of the hydrolysis of lignocellulosic materials. These carbohydrates can be used by many oleaginous yeasts isolated from the soil or other natural sources to produce lipid oils. The objective of this work was to isolate, select and identify oleaginous yeasts isolated from the soil using the glycerol enrichment approach. Also, the lipid producing capability of oleaginous yeasts is investigated with sugar cane molasses and oil palm empty fruit bunches (OPEFB).

3.2. OBJECTIVES

Isolate, select and identify yeasts with high potential to produce microbial oil.

3.2.1 SPECIFIC OBJECTIVES

3.2.1.1 Isolate and select oleaginous yeasts from different environments.

3.2.1.2 Identify by molecular biology at least 10 yeasts with potential for microbial oil production.

3.2.1.3 Evaluate the potential of *Rhodotorula glutinis, Rhodotorula mucilaginosa*, *Rhodotorula s*p. and *Candida tropicalis* on different carbon sources

3.3 MATERIALS AND METHODS

3.3.1 SCREENING AND IDENTIFICATION OF OLEAGINOUS YEAST

3.3.1.1 YEAST GROWTH FROM SOILS

The first stage of the present study was focused on isolating of the yeasts, utilizing glycerol and glucose as carbon source. Soil, sludge, wood, cheese and flowers, samples were obtained for the isolation of yeasts. The samples were collected

in the southern region of Parana, Brazil, where the climate is humid subtropical. For this purpose, approximately 1 g of samples was added into 50 mL of glycerol-enriched medium containing (in g/L): glycerol (50), $(NH_4)_2SO_4$ (1), KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.5), and yeast extract (0.2) in a 250-mL Erlenmeyer flask, and incubated in an shaker at 28 °C for 24 h with shaking at 120 rpm, so that the targeted yeasts from the soil would be enriched to a greater number, modified methodology based on Pan et al. (2009).

3.3.1.2 ISOLATION OF LIPIDS-PRODUCING YEASTS

A volume of 1 mL of the above pre-cultured yeasts was added to 9 mL of distilled water and 10-fold serial dilutions were made. Portions of 0.1 mL from each dilution ranging from 10^{-1} to 10^{-5} were spread onto plates made with dextrose (10 g/L), mycological peptone (5 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), agar (15 g/L), Rose bengal (0.05 g/L) and chloramphenicol (0.10 g/L). The plates were incubated at 28 °C for 2 to 4 days, and those containing isolated colonies with the morphology typical of yeasts were used for further study. Yeast colonies developing on agar medium were picked up, sub-cultured and purified.

3.3.1.3 SELECTION OF OLEAGINOUS YEASTS

In the second step, the isolated colonies of yeasts were further screened for their lipid-producing abilities by quantitative analysis determining lipids content. The oleaginous yeast colonies were initially streaked onto YMA and grown for 2 days. After that they were transferred to 250-mL Erlenmeyer flasks containing 25 mL of inoculation medium containing (in g/L): glucose (15), $(NH_4)_2SO_4$ (5), KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.5), and yeast extract (0.5) and grown at 30 °C on a rotary shaker at 120 rpm for 1 day (Pan et al., 2009). In flask cultures, 10 mL of these cultures were transferred to 90 mL of nitrogen-limited fermentation medium containing (in g/L): glucose (30), $(NH_4)_2SO_4$ (2), KH_2PO_4 (7), NaH_2PO_4 (2), $MgSO_4 \cdot 7H_2O$ (1.5) in 250-mL Erlenmeyer flask, and incubated in a rotary shaker at 120 rpm and 30 °C for 4 days. Isolated yeasts having a higher lipid concentration were selected and then used for subsequent experiments. The screening experiments were performed in sterile media under sterile culture conditions.

3.3.1.4 DRY BIOMASS

Cells from a 50 mL culture growth medium were collected by centrifugation and washed twice with distilled water. Cell dry weight was obtained after drying at 60°C to constant weight. All experiments were performed in triplicate.

3.3.1.5 LIPID ANALYSIS

To determine the lipid content in yeast cells, lipids were extracted, dried and weighed, based on the method of Bligh and Dyer with modifications (1957). This is a fast procedure allowing complete lipid extraction. Briefly, a 50-mL sample was centrifuged at 3500 xg for 15 min, after which the yeast was washed twice with 50 mL of distilled water, then added into 4 mL of 4 M HCl, and incubated at 60 °C for 1 to 2 h. Then the acid-hydrolyzed mass was stirred with 4 mL of chloroform and 8 mL methanol mixture at room temperature for 2 to 3 h, were added 4 mL of chloroform and 4 mL of 1.5% Na_2SO_4 followed by centrifugation at 2000 xg for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. After that the lower phase containing lipids was recovered with a Pasteur pipette and evaporated. The dry lipids were weighed (Pan et al., 2009). The lipid content of the dry material was calculated according to the following formula:

$$LW(g/g) = (m_2 – m_0) \times V /(3 \times m_1)$$

where LW stands by “lipid content based on dry weight,” m_1 is the weight of the yeast, m_0 is the weight of the empty 5 mL glass tube, m_2 is the weight of the 5-mL tube with the dried lipids, and V is the total volume of the lower phase after washing (Zhou et al., 2012).

3.3.2. IDENTIFICATION OF THE BEST LIPID PRODUCING YEAST STRAIN

The identification of the strain was performed by sequencing a fragment of genome. Yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip and resuspended in 50 µL of Ultra-pure water. The suspension was heated for 15 min at 95°C, and 1 µl was used as a DNA template in

PCR experiments. Molecular identification of representative microbial strains was performed by sequence analysis of the full-length internal transcribed spacer (ITS) region. The 55 µL volume reaction consisted of 5.5 µL of 10x PCR buffer (Invitrogen, USA), 2 µL of MgCl2 (50mM), 1.21 µL of dNTP Mix (10mM), 4 µL of the combined forward and reverse primers (ITS5 and ITS4 for yeasts, 27F and 1512r for bacteria), 0.4 µL of 5U.µL-1 Platinum® Taq DNA polymerase (Invitrogen, USA).

The PCR proceeded as the following conditions: initial heating at 95 ºC for 5 min; 30 cycles consisting of 95 ºC for 1min, 50 ºC for 2 min, and 72 ºC for 2 min. Final extension step was made at 72 ºC during 10 min. Amplicons (5 µL) were loaded and separated by electrophoresis in 1.5% agarose gels in 1x TBE at 10 V/cm. DNA markers Gene Ruler 100bp DNA Ladder (Invitrogen, USA) were run along with the samples as reference. After electrophoresis, PCR products were stained with ethidium bromide (5 µg/ml) and scanned using L-PIX HE Transiluminator imaging system (Loccus Biotecnologia, Brasil). The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST).

The entry with the highest sequence similarity was chosen for each isolate. Identities of every isolate determined using the ITS sequences were compared for similarity at genus and species level. Identification to the species level was defined as ≥ 99% ITS sequence similarity to the closest GenBank entry. The isolate was assigned to the corresponding genus when its sequence similarity was ≥ 99% and ≤ 95% (Loong et al., 2013).

3.3.3 BIOMASS AND LIPID PRODUCTION BY OLEAGINOUS YEASTS WITH DIFFERENT SOURCES CARBON

Cultivations of oleaginous yeasts were performed to evaluate biomass production (g/L) and oil content (%) on xylose, glycerol, sugar cane molasses and hydrolysate of OPEFB. Shake flask cultures in 250 mL Erlenmeyer contained 90 ml of carbon sources of 30 g/L xylose, 30 g/L glycerol, 30 g/L molasses 20 g/L of hydrolysate of OPEFB and suplemented with 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO4 \cdot 7H_20$, 0.5 g/L KH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, pH 5.5 described by Tampitak et al., 2015. The cultivations were performed in triplicates. The flasks were inoculated with 10% (v/v) of

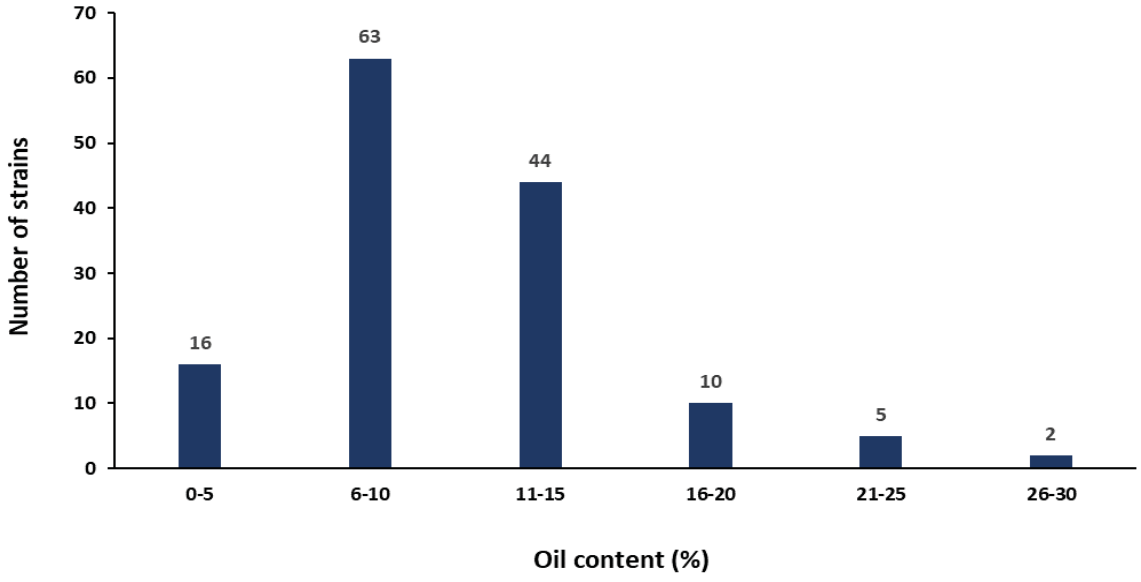
oleaginous yeasts (Candida tropicalis, Rhodotorula sp., Rhodotorula glutinis or Rhodotorula mucilaginosa), which was pre-grown for 48 h in a synthetic medium (25 g/L D-glucose, 10 g/L yeast extract, and 10 g/L peptone, pH 5.5). The flasks were incubated at 30°C for 4 days and 120 RPM. After cultivation, was collected to calculate the yield and lipid concentration.

3.4 RESULTS AND DISCUSSION

3.4.1. THE ISOLATION AND SCREENING OF YEAST COLONIES FROM SOIL SAMPLES

Yeasts were isolated from different environments (soil, sludge, root, cheese, flowers, wood) in the region of Parana, Brazil. Screening of 140 isolated yeast strains revealed seven strains that were found to be highly effective in accumulating oil (up to 20% on dry weight basis) FIGURE. 3.1, as determined by the solvent extraction method (Bligh and Dyer, 1959) probably these can be oil-producing yeasts. Several authors consider yeast as oleaginous when they produce more than 20% of oil with respect to their biomass on a dry basis (Li et al., 2007; Ochsenreither et al., 2016; Ratledge, 2004; Sitepu et al., 2014). Further optimization to explore the potential of these strains in accumulating oil, was attempted.

FIGURE 3.1 - DISTRIBUTION OF LIPID ACCUMULATING ABILITY IN TERMS OF LIPID CONCENTRATION DURING PRIMARY SCREENING



3.4.2 IDENTIFYING OLEAGINOUS YEAST COLONIES

The selected ten oleaginous yeast strains were identified based on its 26S rDNA sequence. The ITS sequence was amplified by PCR using various universal primer sets and sequenced. The obtained sequences were BLAST searched against National Center for Biotechnology Information (NCBI) database. The sequence obtained was compared with those available in the GenBank, and the results showed high sequence similarity (99%) with the type strain of *Rhodotorula sp*. (PPGEBB114), *Rhodotorula glutinis* (PPGEBB115), *Rhodotorula mucilaginosa* (PPGEBB131 and PPGEBB140), *Candida tropicalis (*PPGEBB063) and *Candida palmiolephila* (PPGEBB64). However, only *Pichia Kudriavzevii* (PPGEBB061) presented with an 81 % of similarity (TABLE 3.1).

TABLE 3.1. COLONY CHARACTERISTICS AND SOURCES OF SELECTED YEASTS.

Colony serial number	**Name**	**Source**	**Colony morphology (colony color, size, shape, texture and diameter)**
PPGEBB061	*Pichia Kudriavzevii*	Sludge	White, small, circular, smooth, dry colonies with diameter 2–3 mm
PPGEBB063	*Candida tropicalis*	Sludge	White, small, irregular, wrinkled, moist colonies with diameter of 2–3 mm
PPGEBB064	*Candida palmiolephila*	Soil	White, small, medium, wrinkled, moist colonies with diameter of 2–3 mm
PPGEBB114	*Rhodotorula sp.*	Soil	Orange, small, circular, smooth, moist colonies with diameter 2–3 mm
PPGEBB115	*Rhodotorula glutinis*	Soil	Salmon, small, circular, smooth, moist colonies with diameter 2–3 mm
PPGEBB131	*Rhodotorula mucilaginosa*	Sludge	Pink, small, circular, smooth, moist colonies with diameter 2–3 mm
PPGEBB140	*Rhodotorula mucilaginosa*	Soil	Pink, medium, circular, smooth, dry colonies with diameter 3–5 mm

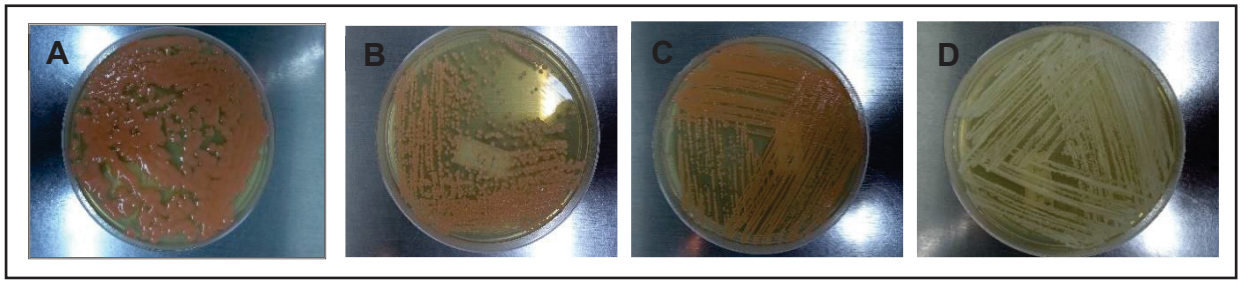
The species of *Rhodotorula glutinis, R. mucilaginosa, Candida tropicalis* and *Pichia Kudriavzevi* are considered oleaginous yeasts and have been studied by several researchers to produce microbial oil (TABLE 3.2).

TABLE 3.2. LIPID CONTENT IN THE CELL BIOMASS OF DIFFERENT YEAST STRAINS

Strain	Cultivation method	Carbon source	Nitrogen source	Lipid content (%)	References
R. glutinis					
L/24-2-1	Single-stage continuous	Molasses	Ammonium sulphate	39	Alvarez et al., 1992.
ATCC 204091	Batch	Distillery wastewaters from the Tequila Production process.	-	27	Gonzalez-Garcia et al., 2013.
CGMCC 2703	Fed-batch	Corncob hydrolysate	Ammonium sulphate	47.2	Liu et al., 2015.
TISTR 5159 R.	Batch	Glycerin fraction after diesel production 36.9		36.9	Louhasakul and Cheirsilp, 2013
R. mucilaginosa					
H3-4	Flask	Xilose	Ammonium sulphate	36.4	Pan et al., 2009.
-	Flask	Molasses	Ammonium sulphate	69.5	Karatay et la., 2010
TJY15a 45.9	Batch	Hydrolysate of cassava starch	Yeast extract	45.9	Andre et al., 2010.
C. tropicalis					
-	Flask	Molasses	Ammonium sulphate	46.8	Karatay et al., 2010.
J31	Flask	Xylose	Ammonium sulphate	24.88	Pan et al., 2009.
Pichia Kudriavzevii					
MTCC 5493	Fed-Batch	Crude glycerol,	Yeast autolysate and corn steep liquor	19	Sankh et al., 2013

Until recently, the yeasts of the genus *Rhodotorula* were primarily considered to be saprophytes that spoil food. *Rhodotorula* species, are characterized by pink, orange, red pigments. They are fast-growing colonies (mature in 4 days), with a size of 2 to 3 cm in diameter morphologically smooth, moist and sometimes mucoid in appearance (Bonifaz, 2012; Larone, 2011), with respect to *Candida tropicalis* is uniform white color, as shown in FIGURE 3.2.

FIGURE 3.2. YEAST COLONIES ON YMA AGAR MEDIUM AFTER 4 DAYS OF INCUBATION: A) *Rhodotorula sp*., B) R. *glutinis*, C) *R. mucilaginosa,* D) *Candida tropicalis.*



Although the *Rhodotorula* species are classified as oleaginous yeasts can synthesize other compounds of industrial interest, in recent times, a large number of studies have been published on the biotechnological uses of these yeasts, which suggest that they may constitute important group of microorganisms that might be of importance in industries in the future. For example, *Rhodotorula glutinis* is considered to be the typical species of this genus. This yeast is capable of synthesizing numerous valuable compounds with a wide industrial usage. Biomass of this yeast constitutes sources of microbiological oils, and the whole pool of fatty acids is dominated by oleic, linoleic, and palmitic acid. Due to its composition, the lipids may be useful as a source to produce the so-called third-generation biodiesel (Kot et al., 2016). These yeasts are also capable of synthesizing carotenoids such as β-carotene, torulene, and torularhodin. Due to their health-promoting characteristics, carotenoids are commonly used in the cosmetic, pharmaceutical, and food industries. They are also used as additives in fodders for livestock, fish, and crustaceans. A significant characteristic of *R. glutinis* is its capability to produce numerous enzymes, in phenylalanine ammonia lyase (PAL). This enzyme is used in the food industry in the production of L-phenylalanine that constitutes the substrate for the synthesis of aspartame sweetener commonly used in the food industry (Kot et al., 2016).

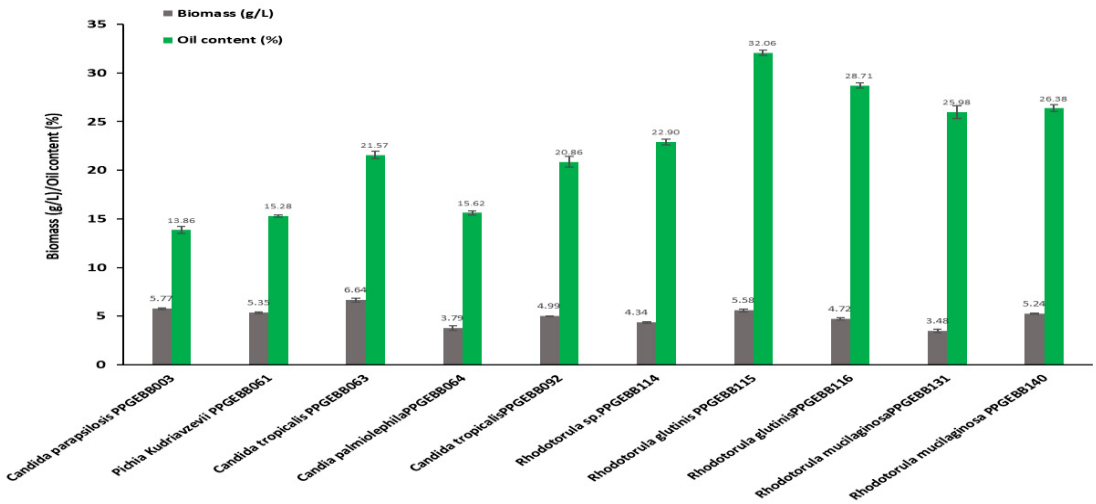
Candida parapsilosis e *Candida palmiolephila* does not exist reference of research works as producers of microbial oil, only works of isolation and molecular identification. With respect to *Pichia kundriavzeii* is a yeast producing alcohol and organic compounds as described in WO 2015194921 A1 (Novel *Pichia kudriavzevii* strain ng7 and use of same), has resistance to heat and acids. Hisamatsu et al., (2006) reported ethanol production at high temperatures of the strain MF-121 exhibited multi-stress tolerance including acid tolerance, ethanol tolerance, thermotolerance and salt

tolerance. This strain of *P. kundriavzeii* produce a large amount of ethanol in acidic media containing salt concentrations, e.g., it produced 2.9 % (w/v) ethanol in medium containing 50 g/L Na_2SO_4 at pH 2.0, or at high temperatures, e.g., it produced 3.1 % (w/v) ethanol at 43 °C (Hisamatsu et al., 2006; Isono et al., 2012). However, the strains of *P. kudriavzevii* that have been studied as oleginous yeast to produce biodiesel, as in the work done by Sankh et al., (2013) using cheap substrates such as crude glycerol, yeast autolysate and corn steep liquor, in a Fed-batch fermentation yielded a dry biomass and oil yield of up to 33 g L^{-1} and 19% (w/w) respectively.

3.4.3 LIPID CONTENT AND FATTY ACIDS OF OLEAGINOUS YEAST

As we have already mentioned, the lipid content and biomass production were determined, we can observe in Figure 3.3, that the yeast with the highest oil content was *R. glutinis* with 31.75%, biomass of 5.58 g/L and lipid productivity of 0.018 g/L.h, followed by *R. mucilaginosa* with 25.98 %, *Rhodotorula sp*. 22.21% and C. tropicalis 21.57%. The yeast oil content depends on several factors from the genetics of the species it self to the factors influencing a fermentation process such as: type of susbtrato, nitrogen source, aeration rate, disolved (DO), pH, temperature, C / N ratio, among others. For example, we can find that several research works using the species of *Rhodotorula glutinis* can produce oil from 9.1% to 66% (Gientka et al., 2017; Alvarez et al., 1992; Dai et al., 2007; Easterling, et al., 2009; Gonzalez-Garcia et al., 2013; Johnson et al., 1992, Johnson et al., 1995, Lee and Yoon, 1990, Li et al., 2013, Liu et al., 2015; Lopes da Silva et al., 2011, Louhasakul and Cheirsilp, 2013, Ratledge and Hall, 1979, Saenge et al., 2011b; Wang et al., 2009, Xue et al., 2008; Xue et al., 2010; Yen et al., 2012; Yen and Chang, 2014; Yu et al., 2011), which will also depend on the type of cultivation mode used as flask, bacht or Fed batch.

FIGURE 3.3. BIOMASS PRODUCTION (G/L) AND OIL CONTENT OF 10 YEASTS, USING 3 % GLUCOSE, 0.11 G/L YEAST EXTRACT, 5 G/L $(NH_4)_2SO_4$, AT 30 °C, 4 DAYS.



Regarding the fatty acids of the yeasts identified, they range from C: 14 to C: 20, but the content will depend on the species. For example, *R. glutinis* is composed mainly of palmitic acid (13.3 to 40.52%), stearic acid (0.7 to 10.33%), oleic acid (21.3 to 58.6%), linoleic acid (3.6 to 26.57%), palmitoleic acid (16%), linolenic acid (3.6 to 26.57%) and arachidonic acid (0.3 to 17%); for *R. mucilaginosa* has palmitic acid (15.5 to 26.2%), stearic acid (5 to 37.3%), oleic acid (22.3 to 63.5%), linoleic acid (5.7 to 15.8%) and arachidonic acid (0.7 to 1.5); *Candida tropicalis* has palmitic acid (20.37 to 29.7%), stearic acid (10.33 to 56.2%), oleic acid (22.3 to 47.78), linoleic acid (1.5 to 7.31%) and linolenic acid (0.55 to 0.98%); and finally *Pichia kundriavzeii* (although in this species there is little research) has the following fatty acids: stearic acid (1.0 to 8.89%), palmitic acid (10 to 29.3%), oleic acid (41 to 41.9%), linoleic acid (9.5 to 9.22), linolenic acid (4.3 to 6%).

Profile of fatty acids synthesized by oleaginous yeasts primarily depends on the yeast strain and composition of the culture medium (Zhang et al. 2011). However, the composition of the lipids can also be adjusted by modifying the molar ratio C/N in the culture medium (Braunwald et al. 2013), temperature of cultivation (Suutari et al. 1990), and by genetic modification of yeast (Shichang et al. 2013). A significant impact on the profile of fatty acids synthesized also depends on time of cultivation (Mast et al., 2014; Zhang et al. 2011). Zhang et al. (2011) noted that increasing the time of cultivation *R. glutinis* ATCC 15,125 yeast increased content of unsaturated fatty acids, from 46 (0 h) to 63.1 % (233 h). Now, oleic and linoleic acids content increased from 26.9 and 8.5 to

43.8 and 12.7 %, respectively. Composition of fatty acids synthesized is influenced by temperature of cultivation. Changes in the proportions of fatty acids are one of the factors of yeast adaptation to life in environments with different temperatures. At a lower temperature, yeasts synthesize more unsaturated fatty acids, which is associated with changes of the cell membranes (Zlatanov et al. 2010).

With these characteristics of the oil content and the composition of fatty acids previously discussed, we can that isolated oleaginous yeasts represent a high potential for the production of biodiesel due to their composition of fatty acids that directly influence the quality of biodiesel in some properties, such as cold filter plugging point (CFPP), flash point (FP), cetane number (CNV), low calorific value (LCV) and kinematic viscosity (m) as described by Leiva-Candia et al. (2014) in their publication.

3.4.4. THE ABILITY OF SEVERAL OLEAGINOUS YEASTS TO ASSIMILATE DIFERENTS SOURCES OF CARBON

At present, there is the interest to produce microbial oil from oleaginous yeasts using low cost substrates such as agroindustrial waste. In this work the assimilation capacity of four isolated yeasts (*R. glutinis, Rhodotorula sp., R. mucilaginosa and C. tropicalis*) was evaluated using xylose, glucose, sugar cane molasses, glycerol and hemicellulose hydrolysate of oil palm empty fruits bunches (OPEFB) determining biomass production (g / L) and oil content (%). As can be seen in Table 3.3, they grow better in glucose than in xylose and glycerol which are synthetic carbon sources. While that in agroindustrial residues, the yeasts present a greater assimilation in sugar cane molasses than in the OPEFEB hydrolyzate. The low yeast growth in the OPEFB hydrolyzate is due to the composition of sugars (xylose, glucose, arabinose, cellobiose) and inhibitory compounds (acetic acid, formic acid, HMF and furfural) during acid hydrolysis which affecting the growth and oil production some yeast species, as mentioned by Tampitak et al., (2015). We can see that *Rhodotorula glutinis* is one of the species that has greater potential in the oil production using different carbon sources, only presents a low productivity 0.008 g / L / h when using OPEFB for its growth, perhaps because there is an effect negative reaction of the OPEFB hydrolyzate compositions its development during the fermentation process. Also the

process conditions must be improved to meet its potential. Later in chapter II, we will discuss the effect of the OPEFB hydrolysate on the growth and production of lipids.

TABLE 3.3. INFLUENCE OF CARBON SOURCE ON OIL PRODUCTION WITH OLEAGINOUS YEASTS.

Carbon source	Microorganisms	Biomass (g/L)	Lipid in medium (g/L)	Lipid in cells (wt.%)	Lipid productivity ($g/L^{-1}h$)
Glucose					
	Rhodotorula glutinis	5.58 ± 0.28	1.79 ± 0.121	32.06 ± 0.548	0.019 ± 0.0013
	Rhodotorula mucilaginosa	5.24 ± 0.15	1.38 ± 0.077	26.38 ± 0.728	0.014 ± 0.008
	Rhodotorula sp.	5.46 ± 0.23	1.21 ± 0.085	22.90 ± 0.629	0.013 ± 0.009
	Candida tropicalis	6.32 ± 0.45	1.43 ± 0.048	21.57 ± 0.742	0.015 ± 0.0005
Xilose					
	Rhodotorula glutinis	4.07 ± 0.11	1.256 ± 0.109	30.845 ± 1.817	0.013 ± 0.0013
	Rhodotorula mucilaginosa	3.76 ± 0.14	0.673 ± 0.032	17.905 ± 0.177	0.007 ± 0.0003
	Rhodotorula sp.	3.92 ± 0.07	1.005 ± 0.041	25.635 ± 0.573	0.010 ± 0.0004
	Candida tropicalis	4.39 ± 0.24	0.681 ± 0.066	15.505 ± 0.658	0.007 ± 0.0006
Cane molasses					
	Rhodotorula glutinis	5.96 ± 0.134	1.968 ± 0.084	33.04 ± 0.672	0.020 ± 0.0009
	Rhodotorula mucilaginosa	5.11 ± 0.170	1.189 ± 0.082	23.26 ± 0.827	0.012 ± 0.0009
	Rhodotorula sp.	5.56 ± 0.311	1.423 ± 0.115	25.59 ± 0.646	0.015 ± 0.0012
	Candida tropicalis	5.74 ± 0.410	1.026 ± 0.069	17.88 ± 0.071	0.011 ± 0.0007
Hydrolysate of OPEFB					
	Rhodotorula glutinis	1.83 ± 0.092	0.53 ± 0.034	29.17 ± 0.396	0.006 ± 0.0004
	Rhodotorula mucilaginosa	5.11 ± 0.255	0.88 ± 0.066	17.26 ± 0.339	0.010 ± 0.0006
	Rhodotorula sp.	2.84 ± 0.262	0.61 ± 0.085	21.8 ± 0.453	0.006 ± 0.0009
	Candida tropicalis	6.8 ± 0.693	0.96 ± 0.067	14.06 ± 0.392	0.010 ± 0.0007
Glycerol					
	Rhodotorula glutinis	2.17 ± 0.247	0.638 ± 0.092	29.42 ± 0.884	0.007 ± 0.0010
	Rhodotorula mucilaginosa	2.35 ± 0.297	0.489 ± 0.085	20.75 ± 0.992	0.005 ± 0.0009
	Rhodotorula sp.	3.71 ± 0.226	1.069 ± 0.078	28.80 ± 0.334	0.011 ± 0.0008
	Candida tropicalis	3.89 ± 0.219	0.587 ± 0.047	15.10 ± 0.346	0.006 ± 0.0005

Several studies have been carried out on the isolation of yeasts with high potential for the production of lipids in different environments. Table 3.4 presents a summary of some papers that have been published in different scientific journals.

TABLE 3.4 SEVERAL PAPERS PUBLISHED ON THE ISOLATION OF OLEAGINOUS YEASTS.

Species of yeast isolated	**Medium culture**			**Operating conditions**	**Lipids content (%)**	**References**
	Carbon	**Nitrogen**	**Others**			
Rhodosporidium toruloides, Trichosporon pullans, Lipomyces starkey, T. cutaneum	Xilose , 40 g/L	Yeast extract, 1 g/L $(NH_4)_2SO_4$ 2 g/L	KH_2PO_4, 7 g/L NaH_2PO_4, 2 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L	180 RPM 28° C 5 days	17.32-38.94	L.X. PAN *et al., 2009*
Candida sp., Candida Tenuis	Glicerol, 30 g/L	Yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L	KH_2PO_4, 7 g/L NaH_2PO_4, 2 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L CaCl, 0.5 g/L $FeCl_3.6H_2O$, 0.15 g/L $ZnSO_4.7H_2O$, 0.02 g/L $MnSO_4.H_2O$, 0.06 g/L	185 RPM 28 ° C 9 days pH 6	31 - 64	Duarte *et al*., 2013
Cryptococcus sp., Rhodotorula sp., Cryptococcus podzoricus	Glicose, 30 g/L	Yeast extract, 0.1 g/L $(NH)_2SO_4$, 5 g/L	$MgSO_4$, 0.5 g/L CaCl, 0.1 g/L NaCl,0.1 g/L	150 RPM 27 ° C 4 days	20 - 60	Tanimura A*. et al .,* 2014
Candida Tropicalis Rhodotorula glutinis Rhodotorula sp. Rhodotorulha mucilaginosa	Glicose, 30 g/L	Yeast extract, 0.1 1 $(NH_4)_2SO_4$, 5 g/L	KH_2PO_4,7 g/L NaH_2PO_4, 2 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L	120 RPM 30 ° C 4 days	3 - 33	In this work 2017

3.5 CONCLUSIONS

In this work 140 yeasts were isolated, of which 10 yeasts were identified by molecular biology. The yeasts *Rhodotorula glutinis* PPGEBB115, *R. mucilaginosa* PPGEBB131, *Rhodotorula sp.* PPGEBB114, *Candida tropicalis* PPGEBB063 and *Pichia kudriavzevii* PPGEBB092 were oil yeast with oil content of 31.72 %, 25.78 %, 22.21 % and 15.28 %, respectively using glucose as the carbon source. The fatty acids present in isolated yeasts show that they have potential to produce biodiesel.

Regarding the evaluation of yeasts on the use of different carbon sources, *Rhodotorula mucilaginosa* PPGEBB115 and *Candida tropicalis* PPGEBB063 presented better production of biomass and lipid content with respect to the other yeasts isolated using OPEFB. *Rhodotorula glutinis* PPGEBB115 presented better production of biomass and oil content with respect to the other yeasts isolated using sugar cane molasse. Which means that yeasts can produce oil using agro-industrial waste, which turns out to be a good alternative.

In the main, we have verified that isolated yeasts have potential as oleaginous yeasts for the production of biodiesel due to their lipid composition, however further studies are needed to optimize the process of each one of them and to achieve high productivity.

4. CHAPTER II: DEVELOPMENT OF A PROCESS TO PRODUCE MICROBIAL OIL USING OIL PALM EMPTY FRUIT BUNCHES (OPEFB) HEMICELLULOSE HYDROLYSATE

ABSTRACT

In recent years, energy crisis and environmental issues such as greenhouse effect, global warming has roused researchers concern. Biodiesel, as renewable energy, has attracted much attention to deal with such problems. This article explores the use of the pretreatment hydrolysate of OPEFB diluted with sulfuric acid for the production of microbial oil. The resulting hydrolysate was composed of pentoses (21.19 g / L) and hexose (0.72 g / L), along with other degradation products, such as acetic acid, formic acid, furfural and hydroxymethyl furfural (HMF). The detoxification studies indicated that using the activated carbon detoxified treatment in the hydrolysate decreased the concentration of the inhibitory compounds considerably, reducing 91.52% HMF, 92.63% furfural, 33.48% acetic acid and 14.48% formic acid. The detoxified hydrolysate presented growth and production of lipids with *Rhodotorula mucilaginosa* 5.11 g/L and 17.26 %, respectively in a flask at 120 RPM. The cell grew well with detoxified hydrolysate in the batch culture of 10 L with C/N ratio of 19.85, lipid yield and lipid content reached 1.78 g/L and 14.62 %, respectively. High cell density culture with two-stage nitrogen feeding strategy was studied to enhance the biomass production, lipid concentration and lipid content of 24.56, 4.85 g/L and 19.75 % were obtained. The results indicated the potential application for lipid production by *R. mucilaginosa* with OPEFB detoxified hydrolysate obtained during pretreatment of lignocellulosic materials.

4.1 INTRODUCTION

Greenhouse gases, mainly carbon dioxide (CO_2), accumulate in the atmosphere and prevent the infrared radiation that the planet emits to heat out into space, this causes the average temperature of the earth to increase. Industry, transport and land use changes increased the concentration of these gases. According to the World Meteorological Organization (WMO), the concentration of CO_2 in the atmosphere in 2015 reached 400 parts per million (ppm), before the Industrial Revolution, was 279 ppm (DeCola and WMO, 2017). Scientists from the Intergovernmental Panel on

Climate Change (IPCC) group showed that if humans continue to rate their emissions without taking steps to reduce them, the global average temperature could rise by 3.7 to 4.8 degrees by 2100, pre-industrial level (IPCC, 2014).

Within the framework of the 21st Climate Change Conference (COP 21), the Paris agreement was formulated, involving 195 member countries. This agreement aims to (a) keep the global average temperature rise far below 2 °C with respect to the levels and to continue efforts to limit this increase in temperature to 1.5 °C with respect to pre-industrial levels, recognizing that this would significantly reduce the risks and effects of climate change; (b) Increase adaptive capacity to adverse effects of climate change and promote resilience to climate and low-greenhouse gas development in a way that does not compromise food production; (c) To raise financial flows to a level compatible with a trajectory conducive to climate-resilient and low-greenhouse gas development (UN, 2017).

Brazil is responsible for producing 2.48% of carbon emissions on the planet according to the Ministry of the Environment (MMA). That is why, in the Paris agreement, it assumed the commitment to limit its annual pollutant emissions to 1, 300.00 million tonnes of carbon dioxide for 2025, which would mean a reduction of 36.1% over 2005. It also offered to maintain that trend and reduce its emissions to 1, 200.00 million tonne of annual carbon dioxide by the year 2030 (MMA, 2016).

One of the alternatives to reducing greenhouse gas emissions (GHG) is the diversification of the energy matrix through the use of biofuels such as biodiesel and bioethanol. Biodiesel is considered a renewable, sustainable and environmentally friendly source of energy and is considered an alternative to substitute fossil fuels (diesel) for the main sources of CO_2. Biodiesel can be obtained by transesterification of vegetable oils and animal fats (Demirbas, 2005) . The cost of production of biodiesel is not economically competitive with petroleum-based fuel, due to the relatively high cost of raw material (Demirbas, 2008; Highlight, 2012). The use of vegetable oils as a raw material for the production of biodiesel competes with edible oils, which leads to an increase in the price of food (Saenge et al., 2011). In Brazil, approximately 75% of biodiesel produced is obtained from soybean oil (Glycine max), the oil content in the seed is around 18%, corresponding to a biodiesel production of 562 kg/ha (Soccol et al., 2017).

Although soybean oil is the main raw material for biodiesel production in Brazil, it presents some drawbacks, such as its use as edible oil and significant price variations in the international market affecting products derived from it. Due to this situation, alternative sources of biodiesel, called "third generation" biodiesel, are being developed, using oil accumulating microorganisms such as microalgae, bacteria, yeasts and fungi (Soccol et al., 2017), these microorganisms promise to be potential raw materials for the production of biodiesel due to its similar composition of fatty acids to that of vegetable oil (Li et al., 2007). The quality of biodiesel depends on the fatty acid composition of the bio-lipids (Knothe and Steidley, 2011). In general, the bio-lipids produced by oleaginous yeasts are suitable raw materials for biodiesel, because the fatty acid composition satisfies important criteria, i.e. the chain length and degree of saturation. However, the composition of the fatty acids in the microbial oil is specific to the strain, therefore, it is important to select strains of oleaginous yeasts to determine their qualifications for the production of biodiesel (Tanimura et al., 2014). Another advantage of yeasts is their ability to produce lipids from unused biomass, including lignocellulosic biomass (Liang et al., 2012; Ryu et al., 2013). Lignocellulosic wastes from forestry, agriculture, and agro-industry are abundant and relatively inexpensive, are not used for food and are attractive as raw materials because they are a rich source of polysaccharides with potential use to produce microbial oils. In general, it can be assumed that the lignocellulosic raw materials contain approximately 40% bound carbon as cellulose, 30% as lignin and 26% as hemicelluloses, and the rest of other polysaccharides (Jönsson and Martín, 2016).

At present, the palm oil (Elaeis guinensis) has considerably increased its production in Brazil, according to the Brazilian Association of Palm Producers (Abrapalma), they have planted more than 235,000.00 hectares in the country, with a production of 2,596,000.00 tonnes of oil palm fruit and average productivity of 11.01 ton/ha destined to the production of oil palm (FAOSTAT, 2017) therefore a production of more than 400,000.00 tonnes of oil is estimated considering 25 % of oil content. At a conservative estimate, for every tonnes of oil produced from oil palm fruit fresh approximately 1 ton of empty fruit bunches (EFB), 0.7 ton of palm fibers, 0.3 ton of palm kernels and 0.3 ton of palm shells are generated, which amounts to a total palm biomass of 2.3 ton (Dalimin, 1995), which would mean that more than 400,000.00 tonnes of oil palm empty fruit bunches (OPEFB) are produced.

The oil palm empty fruit bunches are composed mainly of cellulose (23.7-65 %), hemicelluloses (19.5-33.52 %), Lignin (14.1-32.17 %), extractive (1.17-3.7 %) (Abdullah et al., 2011; Khor et al., 2009; Law, Kwei, Wan, Wan, Ghazali, 2007; Medina et al., 2016; Mohammed et al., 2012; Omar et al., 2011; Shinoj et al., 2010; Sreekala et al., 1997). The OPEFB can be hydrolyzed by acid pretreatment to obtain a mixture of sugars that are potential substrates for the conversion of a wide variety of products, such as microbial oils. A side effect of pretreatment is the formation of by-products derived from lignin that inhibit microbial growth, microbial and enzymatic biocatalysts (Leif J. Jönsson and Carlos Martín, 2016), these are formed due to high Temperatures and low PH. These inhibitors include aldehydes furanic [furfural and 5-hydroxymethylfurfural (HMF)] resulting from degradation of pentose and hexose, respectively; Aliphatic acids (such as acetic acid, which is released from acetyl hemicelulósicos groups, formic acid, are formed when decomposing furfural and levulinic acid, which is obtained when the HMF is degraded) and phenolic compounds (Jönsson and Martín, 2016)

There are different strategies discussed by Leif Jönsson and Carlos Martín (2016) in their review to address the problem of inhibitors formed during the pretreatment under acidic conditions, one of them is the method of detoxification or conditioning of lignocellulosic hydrolysates and slurries, this method is one of the most powerful ways of counteracting inhibition problems as reviewed by Pienkos and Zhang (2009) and Jönsson et al. (2013). This strategy includes techniques using chemical additives, for example, alkali, reducers, and polymers. Other possibilities are the use of enzymatic treatment, heating and vaporization, liquid extraction and liquid-solid extraction (Jönsson et al., 2013). Liquid-solid extraction covers techniques such as ion exchange and treatment with activated carbon (Carvalheiro et al., 2005).

4.2 OBJECTIVES

Develop a process to produce microbial oil from OPEFB using *Rhodotorula mucilaginosa.*

4.2.1 SPECIFIC OBJECTIVES

4.2.1.1 Develop a detoxification process to minimize the toxic compounds obtained during the acid hydrolysis of OPEFB.

4.2.1.2 Optimize a process to produce microbial oil from *Rhodotorula mucilaginosa* using hydrolysate of OPEFB.

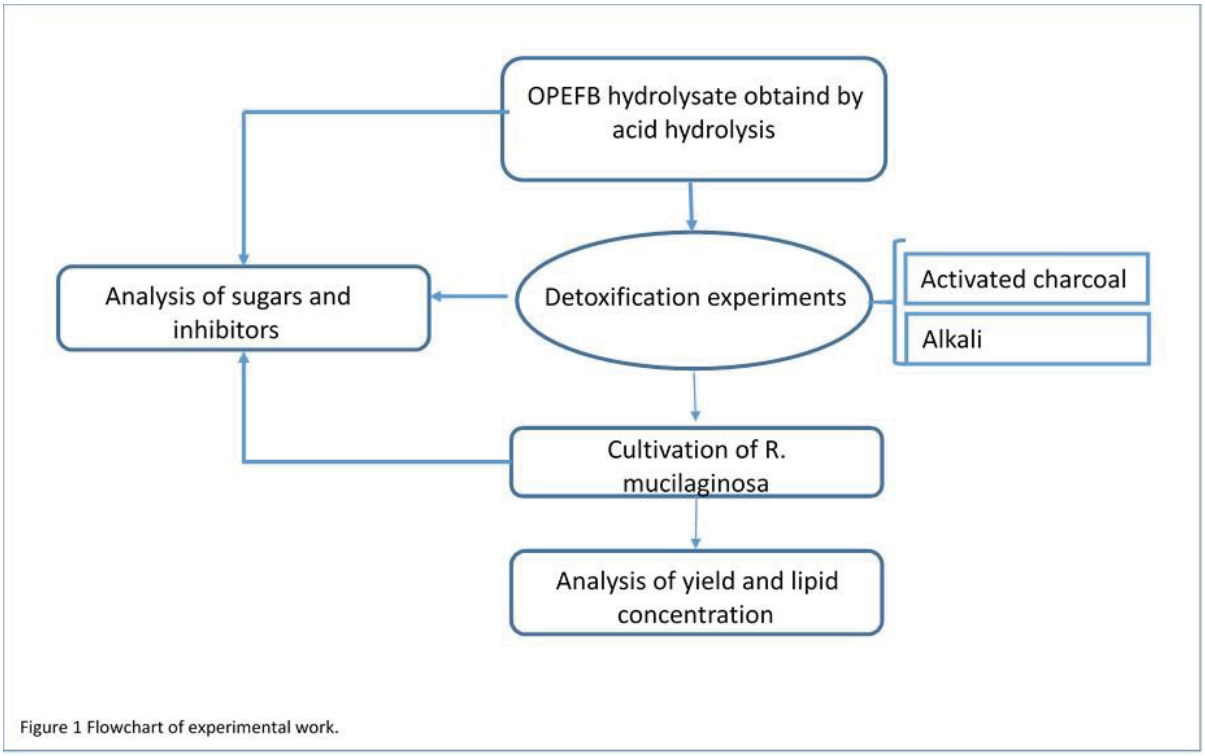
4.2.1.3 Optimize a process to produce microbial oil in a 10-liter reactor from Rhodotorula mucilaginosa using hydrolyzate of OPEFB.

4.3 MATERIALS AND METHODS

4.3.1 RAW MATERIAL AND COMPOSITION OF OPEFB

An overview of the investigation is presented in Figure 4.1. Oil palm empty fruit bunches (OPEFB) were obtained from Biopalma Vale factory, located in Mojú, Pará, state of Brazil. The OPEFB was dried in a cross-flow stove at 65 °C for 72 h and stored in polyurethane bags at room temperature to avoid biological degradation.

FIGURE 4.1- FLOWCHART OF EXPERIMENT WORK.



4.3.2 OPEFB HYDROLYSATE PREPARATION

Oil palm empty fruit bunches was smashed inter 0.84-0.35 mm and then mixed with dilute sulphuric acid (1.5%, w/w) to give a mixture with a solid loading of 10% (w/w). The mixture was treated in an autoclave at 125 °C for 15 min and the liquid fraction was separated by vacuum filtration after cooling and stored at 4 °C prior to use.

4.3.3 DETOXIFICATION PROCEDURES

Detoxification procedures were carried out over raw or pH-adjusted hydrolysates as required. In the later, pH was raised to 5.5 (fermentation pH) by the addition of $Ca(OH)_2$ and NHOH. For treatment wiht Ca $(OH)_2$ after 1 h at pH 5.5 the precipitate was removed by centrifugation at 4500 x g for 25 min.

4.3.3.1 TREATMENT WITH ACTIVATED CHARCOAL

Granular activated charcoal 2.5 mm was washed with water and equilibrated with HCl 0.4 M, washed again with water and dried at room temperature. Charcoal was mixed with the pH 5.5 hydrolysates (1:10 (w/v)) and stirred for 1 h at room temperature. The detoxified hydrolysates were recovered by vaccum filtration (Whatman no. 1 filter paper). When needed the pH of treated hydrolysates was corrected to 5.5 with $Ca(OH)_2$ or H_2SO_4 as described by Carvalheiro et al. (2003).

4.3.3.2 TREATMENT WITH ÁLCALI

Alkali treatments of the spruce hydrolysate were performed (i) by adding $Ca(OH)_2$ to pH 10.0 and incubating for 1 h at 30°C after were centrifuged at 4500 x g for 25 min, acidified to pH 5.5 with 98% (w/w) H_2SO_4 described by Carvalheiro et al. (2003). or (ii) by adding 28% NH_4OH to pH 9.0 and incubating for 3 h at 55°C. After the incubation, the hydrolysate was centrifuged at 4500 g for 10 min and the supernatant was then adjusted to pH 5.5 with H_2SO4 described by Guo et al. (2013).

4.3.4 Analysis of sugars and inhibitors

The cellulose and hemicellulose mass composition were calculated using the equations reported by Tan et al. (2013), in which the concentration of glucose, xylose and arabinose are correlated. Analysis of acid hyrolysate was carried out by High-Performance Liquid Chromatography (HPLC), in a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working at 60 °C with sulfuric acid (5 mmol L^{-1}) as mobile phase at flow rate of 6 mL min^{-1}.

Furfural (F), hydroxymethyl furfural (HMF), acetic, formic and levulinic acid were determined in the pretreatment hydrolyzates using a Shimadzu Chromatograph equipped with an Aminex HPX-87H and C18 columns at 60 °C. Mobile phase used was sulfuric acid (5 mmol L^{-1}) at rate of 6 mL min^{-1} with an IR detector. Detection was carried out by differential refractometry and the quantification was based on external calibration as described by Scholl et al. (2015a).

4.3.5 CULTIVATION OF *Rhodotorula mucilaginosa* WITH POEFB HYDROLYSATE

Cultivations of *Rhodotorula mucilaginosa* were performed to evaluate the effectiveness of the detoxification treatments and an untreated hydrolysate (control hydrolysate). Shake flask cultures in 250-mL Erlenmeyer flasks contained 100 mL of hydrolysate based medium (20.26 g/L xilose, 0.22 g/L glucose, 1.73 g/L arabinose, 0.44 g/L celobiose) and suplemented with 2 g/L $(NH_4)_2SO_4$, 3 g/L $MgSO_4 \cdot 7H_20$, 0.5 g/L KH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, pH 5.5 described by Tampitak et al., 2015. The cultivations were performed in triplicates. The flasks were inoculated with 15% (v/v) *Rhodotorula mucilaginosa*, which was pre-grown for 48 h in a synthetic medium (25 g/L D-glucose, 10 g/L yeast extract, and 10 g/L peptone, pH 5.5). The flasks were incubated at 30°C for 4 days and 120 RPM. After cultivation, was collected to calculate the yield and lipid concentration. The concentrations of glucose, xylose, mannose, arabinose, galactose, formic acid, acetic acid, furfural, and HMF were determined.

4.3.6 BIOMASS, OIL CONTENT AND FATTY ACIDS

4.3.6.1 DRY BIOMASS

To determine biomass, culture broth was harvested by centrifugation at 3,500 xg for 15 min. The cell pellets were washed twice with distilled water and then oven-dried at 60 °C to get constant weight. The biomass, expressed as dry cell weight (DCW), was determined gravimetrically (Poontawee et al., 2017). All experiments were performed in triplicate.

4.3.6.2 LIPID ANALYSIS

To determine the lipid content in yeast cells, lipids were extracted, dried and weighed, based on the method of Bligh and Dyer with modifications (1957). This is a fast procedure allowing complete lipid extraction. Briefly, a 50-mL sample was centrifuged at 3500 xg for 15 min, after which the yeast was washed twice with 50 mL of distilled water, then added into 4 mL of 4 M HCl, and incubated at 60 °C for 1 to 2 h. Then the acid-hydrolyzed mass was stirred with 4 mL of chloroform and 8 mL methanol mixture at room temperature for 2 to 3 h, were added 4 mL of chloroform and 4 mL of 1.5% Na_2SO_4 followed by centrifugation at 2000 xg for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. After that the lower phase containing lipids was recovered with a Pasteur pipette and evaporated. The dry lipids were weighed (Pan et al., 2009). The lipid content of the dry material was calculated according to the following formula:

$$LW(g/g) = (m_2 - m_0) \times V / (3 \times m_1)$$

where LW stands by “lipid content based on dry weight,” m_1 is the weight of the yeast, m_0 is the weight of the empty 5 mL glass tube, m_2 is the weight of the 5-mL tube with the dried lipids, and V is the total volume of the lower phase after washing (Zhou et al., 2012).

4.3.6.3 ANALYSIS FATTY ACIDS

The lipid composition was determined by gas chromatography (GC-2010) with ionization detector and a Varian capillary column CP-FFAP CB (25 m, 0.32 mm, 0.3

lm # CP 7485). The column temperature was maintained at 180 °C for 5 min and upgraded from 180 to 250 ° at a rate of 10 °C/min and kept for 6 min. Glyceryl triheptadecanoate was used as internal standard.

4.3.7 OPTIMIZATION OF CULTURE CONDITIONS FOR LIPID PRODUCTION

The effects of initial pH on lipid accumulation were investigated at pH values of 5, 5.5 and 6 in the OPEFB hidrolysate medium suplemented with 2 g/L $(NH_4)_2SO_4$, 3 g/L MgSO4·7$H_2$0, 0.5 g/L KH_2PO_4, 0.1 g/L $CaCl_2$·2H_2O, pH 5.5. To examine the effect of initial nitrogen concentrations on lipid accumulation the cultures were grown in OPEFB hydrolysate media containing 0.75, 1.0, 1.5 and 2 g/L $(NH_4)_2SO_4$ and suplemented with 3 g/L $MgSO_4$·7$H_2$0, 0.5 g/L KH_2PO_4, 0.1 g/L $CaCl_2$·2H_2O, pH 5.5, at pH 5.5. *Rhodotorula mucilaginosa* was cultivated in the media that supplying the optimum conditions for four days. Each of the experiments and measurements described below were performed in triplicate to follow the changes in the samples throughout the incubation period.

4.3.8 Bioreactor fermentation

4.3.8.1 MICROORGANISM AND MEDIUM

The seed medium containing 15 g/L glucose, 2 g/L $(NH_4)_2SO_4$, 1 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L Na_2HPO_4, and 1.5 g/L $MgSO_4$, pH 5.5. The OPEFB hydrolysate, supplemented with 2.5 g/L $(NH_4)_2SO_4$,1 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L Na_2HPO_4, 3 g/L $MgSO_4$, 0.1 g/L $CaCl_2$ and 0.12 g/L $FeCl_3$, was used as the basic fermentation medium, pH 5.5, medio utlizado con base a los resultados de optimizacion.

4.3.8.2. SHAKE FLASK CULTURE

Seed was cultured in 500 mL shake flask containing 100 mL medium at 30 °C and 120 rpm for 48 h when the OD 600 reach 3.0. Fermentation in shake flask was conducted at 30° C and 120 rpm. The inoculation size was 10% (v/v).

4.3.8.3 BIOREACTOR FERMENTATION BATCH AND FED BATCH

Batch fermentation was carried out in 10 L fermenter (BIOFLO 110) with working volume of 3 L, the temperature was maintained at 30 °C, 1.5 vvm and pH was not controlled. For fed-batch culture, concentrated detoxified hydrolysate were fed repeatedly to remain the sugar concentration at around 15 g/L and $(NH_4)_2SO_4$ and yeast extract were fed for the first stage and only concentrated hydrolysate were fed for the second and third stage. The pH was controlled at 5.5.

4.4 RESULTS AND DISCUSSION

4.4.1 CHEMICAL COMPOSITIONS OF UNTREATED OPEFB, NON-DETOXIFIED LIQUID HYDROLYSATE (NDLH) AND DETOXIFIED LIQUID HYDROLYSATE (DLH)

The chemical compositions of the untreated OPEFB products are shown in TABLE 4.1**.** This contains 28.1% hemicellulose and 34.9% cellulose**.** The structure of the untreated OPEFB can be observed in figure 4.1. Cellulose is the main structural component in the cell walls of plants and has an organized fibrous structure. The long chains of the cellulose polymers are linked together by hydrogen and Vander Waals, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils (Kumar et al., 2009). The cellulose structure is shown in the Fig. 4.1a. The main characteristic that distinguishes the cellulose hemicellulose is that the hemicellulose has branches with chains formed by different sugars (Fig. 4.1b). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1,4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds (Kuhad et al., 1997).

TABLE 4.1 CHEMICAL COMPOSITION OF OPEFB ON DRY SOLID BASIS.

Compositions	**Dry solids (%, w/w)**
Hemicellulose	28.1
Celulose	34.9
Lignina	15.93
Cinzas	3.35
Outros	17.72

These monosaccharides include pentose, hexose and uronic acids. In contrast to cellulose, the polymers present in hemicelluloses are readily hydrolysable (Kumar et al., 2009). Lignin, as shown in Fig. 4.1c, is basically found in the primary cell wall,

provides structural support, contains cross-linked polymers of phenolic monomers, impermeability and resistance against microbial attack (Béguin and Aubert, 1994; Kumar et al., 2009; Medina et al., 2015; Pérez et al., 2002).

FIGURE 4.2 - STRUCTURE OF OIL PALM FRUIT FRUIT BUNCHES (OPEFB) COMPOSED OF LIGNIN (A), HEMICELLULOSE AND CELLULOSE.

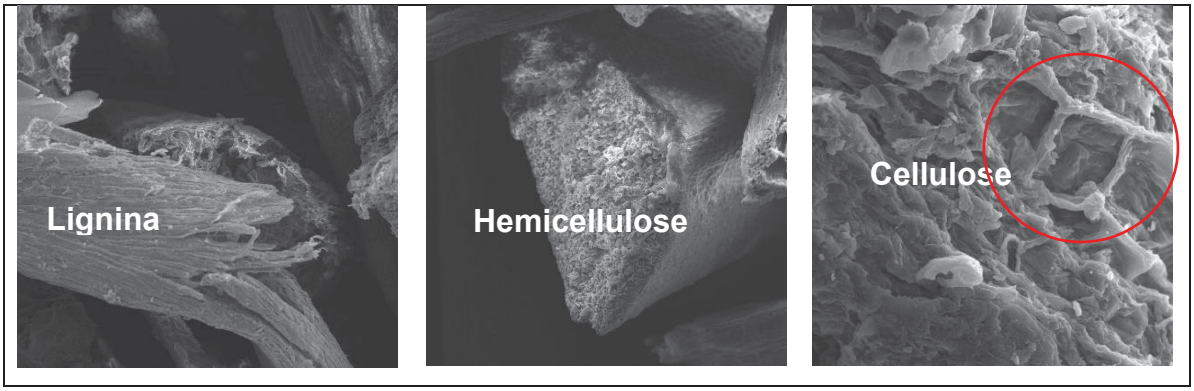


TABLE 4.2, presents the chemical compositions of the non-detoxified liquid hydrolysate and the effect of detoxification methods on the removal of inhibitors in the liquid hydrolysate of OPEFB. After the dilute sulfuric acid pretreatment, sugar monomers from most of hemicellulose dissolved in the hydrolysate with a total liquid yield of 75.01% (w/w). The main objective of the pretreatment is to modify the structure of the lignocellulosic biomass (Fig. 4.1), to separate lignin and hemicellulose, to reduce the crystalline cellulose and to improve the porosity of the materials (Kumar et al., 2009).

During acid hydrolysis, not only fermentable sugars but also various by-products are formed and released into the hydrolysates (Poontawee et al., 2017). These compounds could have negative effects on the growth, metabolism, and formation of microorganism products (Huang et al., 2012; Palmqvist and Hahn-Hfagerdal, 2000; Sitepu et al., 2014). The inhibitory compounds present in the lignocellulosic hydrolysates can be classified based on their origin in three main groups, weak acids, furan derivatives and phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000). However, the concentrations of these compounds depend on the raw material and on the pretreatment and hydrolysis processes (Sitepu et al., 2014).

FIGURE 4.3. STRUCTURE OF OPEFB BEFORE (A) AND AFTER (B AND C) ACID HYDROLYSIS WITH 1.5 % H_2SO_4 AT 120 ° C AND 15 MIN.

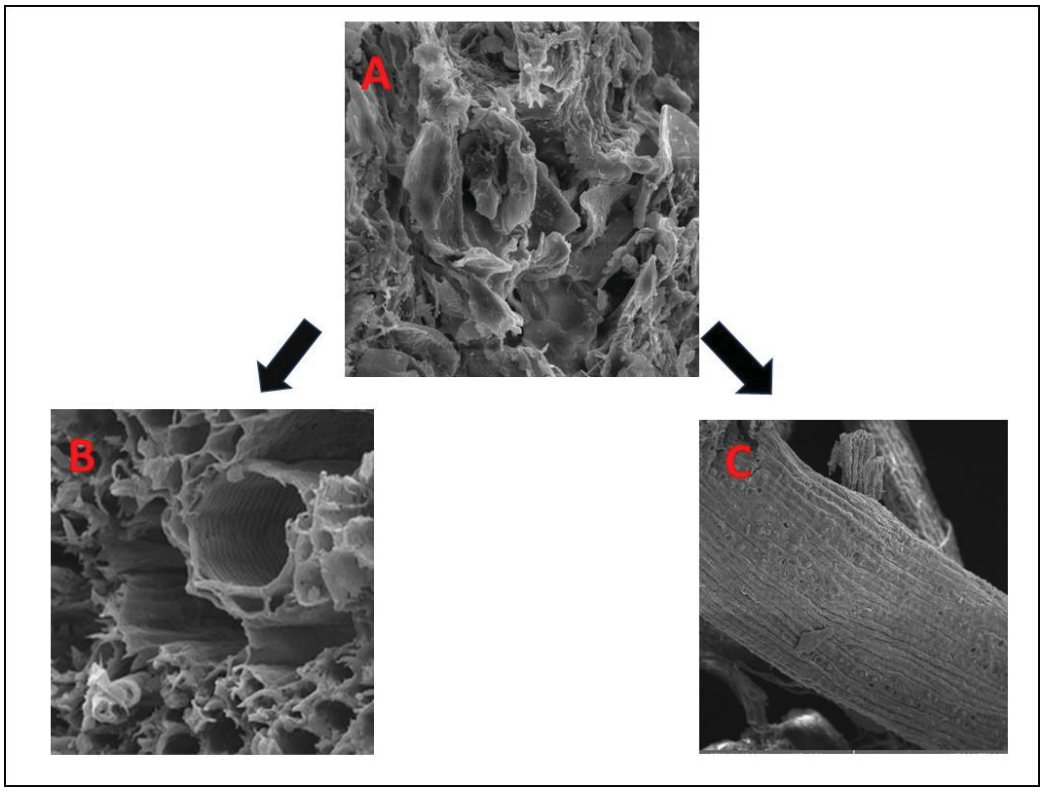


TABLE 4.2. EFFECT OF DETOXIFICATION TREATMENTS ON THE COMPOSITION OF OPEFB HYDROLYSATE.

Composition	Non-detoxified liquid hydrolysate (NDHL) (g/L)	Detoxification treatment (g/L)				
		$Ca(OH)_2$	NH_4OH	Overliming $Ca(OH)_2$	Overliming NH_4OH	Activated charcoal
Glucose	0.21 ± 0.01	0.19 ± 0.29	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.17 ± 0.01
Xylose	19.54 ± 0.71	17.29 ± 0.08	17.48 ± 0.13	17.99 ± 0.06	18.86 ± 0.41	17.23 ± 0.30
Celobiose	0.50 ± 0.09	0.48 ± 0.12	0.47 ± 0.02	0.48 ± 0.03	0.48 ± 0.01	0.47 ± 0.01
Arabinose	1.65 ± 0.12	1.37 ± 0.01	1.42 ± 0.07	1.45 ± 0.02	1.37 ± 0.52	1.55 ± 0.03
Total sugars	21.90 ± 0.67	19.33 ± 0.08	19.55 ± 0.17	20.10 ± 0.05	20.90 ± 0.94	17.14 ± 0.23
Acetic acid	4.90 ± 0.16	4.82 ± 0.23	4.94 ± 0.05	4.89 ± 0.02	5.17 ± 1.02	3.26 ± 0.18
Formic acid	1.03 ± 0.08	0.98 ± 0.01	0.98 ± 0.02	0.95 ± 0.07	1.02 ± 0.03	0.88 ±0.02
HMF	0.06 ± 0.04	0.07 ± 0.01	0.06 ± 0.01	0.05 ±0.01	0.05 ± 0.01	0.01 ± 0.01
Furfural	0.23 ± 0.03	0.21 ± 0.01	0.21 ± 0.01	0.14 ±0.00	0.17 ± 0.02	0.017 ± 0.02

The main monosaccharides found in the non-detoxified and detoxified OPEFB hydrolyzate were glucose, xylose, arabinose and cellobiose. In addition to sugar monomers, the next most abundant compound in the hydrolysates was acetic acid formed via the de-acetylation of hemicellulose (Yu et al., 2011), followed by formic acid formado por la degradación adicional del furfural que es inestable en el dehydrative médium (Danon et al., 2013). Furfural and HMF, which resulted from the degradation of pentoses and hexoses, respectively, were detected in low concentrations. The concentration of the pentoses (xylose and arabinose) represents 96.71% of the total sugars obtained in the non-detoxified hydrolysate of OPEFB, which is due to the removal of most of the hemicellulose during dilute sulfuric acid pretreatment. Five types of detoxification methods were used in the OPEFB hydrolyzate. Each potent inhibitor was reduced using either method. After detoxification by the different treatments applied, monosaccharides, acetic acid, formic acid, and furfural were maintained in the hydrolyzate to a lesser extent depending on the detoxification method employed, HMF was present in extremely low concentrations. The best detoxification treatment was activated carbon treatment had the highest effect on inhibitory compounds by removing acetic acid (33.5 % of 4.90 g/L), formic acid (14.46 % of 1.03 g/L), HMF (93.5 % of 0.065 g/L) and furfural (92.63 % of 0.23 g/L) and loss of sugars 21.69 %. In the work done by Tampitak et al., (2015), they used a pretreatment of acid hydrolysis with 0.5% (w/v) H_2SO_4 using OPEFB lignocellulosic biomass, and then applied two methods of detoxification (Overliming with CaOH and carbon activated) showed that the best treatment for the removal of inhibitors was activated carbon there was a reduction of 68% of 0.22 g / L of Furfural and 44% of 0.09 g / L of HMF. With respect to acetic acid and formic acid, they do not mention the formation of these acids during pretreatment. Also Tampitak et al., (2015) reported that the growth of cinco levaduras (*Rhodotorula mucilaginosa G43, K. marxianus X32, C. tropicalis X37 and Rhodotorula glutinis TISTR 5159*) was limited in non-detoxified OPEFB hydrolysate due to the presence of furfural and HMF at concentrations of 0.22 and 0.09 g/L, respectively. The yeast grew faster and better in the detoxified OPEFB hydrolysate. According Huang et al (2009), fermentation of rice straw hydrolysate by Trichosporon fermentans without detoxification gave a poor lipid yield of 1.7 g/l, which was much lower than the result with glucose or xylose as the single carbon source (13.6 g/l or 9.9 g/l). The detoxification pretreatment, including overliming, concentration, and adsorption improved the fermentability of rice straw hydrolysate significantly by removing the

inhibitors. The work done by Liu et al. (2015), showed that in the corncob hydrolyzate only adjusting its pH to 6, Rhodotorula glutinis CGMCC 2.703 had a better oil production compared to a hydrolyzate detoxified by overliming, of 23.5% and 18.2%, respectively. Therefore, applying a detoxification treatment of a lignocellulosic hydrolyzate will depend on the species of the yeast, the resistance of the microorganism on the inhibitory compounds, the lignocellulosic residue type, the pretreatment and the operating conditions employed during the hydrolysis, for to define if it is necessary to add a further process in obtaining microbial oil from oleaginous yeasts, because it would mean an increase in production costs. According to studies carried out by several researchers, activated carbon is one of the treatments that has had greater efficiency in the removal of the toxic compounds used for various types of hydrolysates and that is economically more viable than other types of detoxifidied treatments that could have costs higher in the production process of oil, ethanol or other organic compounds (TABLE 4.3).

TABLE 4.3. EFFECT OF DETOXIFICATION TREATMENTS ON THE COMPOSITION OF DIFFERENT TYPES OF HYDROLYSATES

(continua)

Lignocellulose material hydrolysate	**Detoxification**	**Decrease in hydrolysate composition (%)**	**Microorganisms**	**Bioproduct**	**References**
Sugarcane Bagasse	Neutralization	Sugars (21.2 %), inhibitors data not shown	*Candida shehatae NCIM 3501*	Ethanol	Chandel *et al*., 2007 a
	Overliming	Sugars (14.8 %), inhibitors data not shown.			
Saccharum spontaneum	Overliming	Total reducing sugars (7.66 %), removal furfurals (41.75 %), phenolics (33.21 %).	*Pichia stipitis NCIM3498*	Ethanol	Chandel *et al.,* 2011 a
Brewery's spent grain	Activated charcoal	Total reducing sugars (13 %), removal of furfurals (92 %), HMF (68 %), acetic acid (17 %), formic acid (11 %), levulinic acid (100 %).	*Debaryomyces hansenii CCMI 941*	Xylitol	Carvalheiro et al 2005
	Anion exchanger	Total reducing sugars (7 %), removal of furfurals (64 %), HMF (44 %), acetic acid (48 %), formic acid (58%), levulinic acid (100 %).			
Sugar cane bagasse	Overliming	Total reducing sugars (7.59 %), removal of furfurals (45.83%)	*Trichosporon fermentans*	Ethanol	Huang *et al*., 2012

TABLE 4.3. EFFECT OF DETOXIFICATION TREATMENTS ON THE COMPOSITION OF DIFFERENT TYPES OF HYDROLYSATES

(conclusão)

Lignocellulose material hydrolysate	Detoxification	Decrease in hydrolysate composition (%)	Microorganisms	Bioproduct	References
Oil palm empty fruit bunch fiber (OPEFB)	Overliming	Total reducing sugars data not shown, removal of furfurals (68 %), HMF (44 %).	*Candida tropicalis*	Biolipids	Tampitak *et al*., 2015
	Ion exchange	Total reducing sugars data not shown, removal of furfurals (55 %), HMF (25 %).	*Candida tropicalis*	Biolipids	
Corncob	Activated charcoal	Total reducing sugars (18 %), removal of furfurals (100 %), HMF (80 %), acetic acid (100 %)	*Rodotorulha glutinis*	Biolipids	Liu et al 2015
Oil palm empty fruit bunch fiber (OPEFB)	Activated charcoal	Total reducing sugars (19 %), removal of furfurals (92 %), HMF (95 %), acetic acid (50 %), formic acid (18 %).	*Rodotorulha mucilaginosa*	Biolipids	This work, 2016

4.4.2 OIL PRODUCTION BY USING HYDROLYSATES

Based on the results obtained it was considered to use *Rhodoturula mucilaginosa* to evaluate the effect of the other detoxified hydrolysates on the production of biomass and oil content.

TABLE 4.4. EFFECT OF DIFFERENT HIDROLYSATES DETOXIFIED OF OPEFB ON CELL GROWTH AND LIPID ACCUMULATION OF *Rhodotorula mucilaginosa*

Detoxification treatment	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Sugar utilized (g/L)	$Y_{x/s}$ [b] (%)	$Y_{oil/s}$ [c] (%)
$Ca(OH)_2$	3.71±0.30	16.73±0.32	0.62± 0.01	14.53±0.01	26.25±0.80	4.39±0.05
NH_4OH	2.55±0.02	15.17±0.28	0.39± 0.08	11.08±0.19	23.00±1.10	3.49±0.01
Overliming $Ca(OH)_2$	3.09±0.36	19.87±0.37	0.61± 0.03	15.05±0.09	20.55±0.52	4.08±0.16
Overliming NH_4OH	1.64±0.11	14.14±0.16	0.23± 0.01	8.61 ± 0.27	19.00±0.90	2.69±0.02
Activated charcoal	5.11±0.23	17.26±0.44	0.88±0.07	15.38±0.32	33.21±0.17	5.73±0.37

NOTA: [a] The culture were performed at 30 °C, pH 5.5 and 120 RPM for 4 days. [b] Biomass yield on sugar consumed. [c] Lipid yield on sugar consumed

The result showed that *Rhodotorula mucilaginosa* non-genetic modified can grow in the presence of the inhibitory compounds (Table 4.4) in any detoxification method used. *R. mucilaginosa* had a higher biomass yield of 5.11 g/L, oil content of 17.26 % and productivity of 0.22 g/L/day when activated carbon was used adjusted to pH 5.5 with sodium hydroxide 10 M. Regarding the other treatments between CaOH and Overliming with CaOH, there was no significant difference with respect to lipid yield 0.62 g / L and 0.61 g / L, respectively.

The treatment that *R. mucilaginosa* presented with less tolerance was the treatment of overliming with NHOH with a lipid yield of 0.23 g/L, perhaps due to the presence of acetic acid (5.17 g/L), formic acid (1.01 g/L), HMF (0.05) and furfural (0.17) inhibit cell growth and lipid accumulation.

In the work done by Poontawee et al (2017), they concluded that presence of the inhibitory compounds formic acid and furfural showed strong inhibitory effects on the biomass production and lipid accumulation by R. fluviale DMKU-SP314, while acetic acid,5-HMF and vanillin had less effect. That is, concentrations of acetic acid greater than 1 g / L inhibit 72% of biomass and 97% of oil; with formic acid when using a concentration greater than 0.25 g / L inhibits 52% of biomass and 91% of oil; for furfuraldehyde from a concentration greater than 0.1 g / L inhibits 53% of biomass and 83% of oil, and finally with HFM using a concentration of more than 16% of biomass and 13% of oil, with respect to a concentration of 10.5 g / L of biomass and 5.9 g / L of oil by R. fluviale DMKU-SP314 after a fermentation process without inhibitors.

Hu et al. (2009) reported that 1 mM furfural (> 0.096 g / L) and> 14.7 mM HMF (> 1.85 g / L) inhibited cell growth and lipid production of *Rhodosporidium toruloides* Y4 oleaginous yeast. They also showed that these inhibitors most likely repressed cell growth more severely than lipid biosynthesis. Depending on the source of the biomass, the type of hydrolysis and detoxification used, the concentration of these inhibitors in lignocellulosic hydrolysates may range from 0.5 to 11 g/L (Almeida et al., 2007). It is believed that these inhibitors damage cell walls and membranes and inhibit RNA synthesis in microorganisms (Heer et al., 2009; Li et al., 2010).

For example, acid hydrolysis of cane bagasse using the following conditions of pretreatment 2.5% HCl and neutralization with excess $Ca(OH)_2$ to pH 6.5 in an autoclave at 121 °C for 45 min produced concentrations of furfural and HMF as high as 0.48 and 0.09 g/L, respectively (Tsigie et al., 2011). In the work done by Tampitak et al. (2015) with hydrolysate of the OPEFB hemicellulose using the following

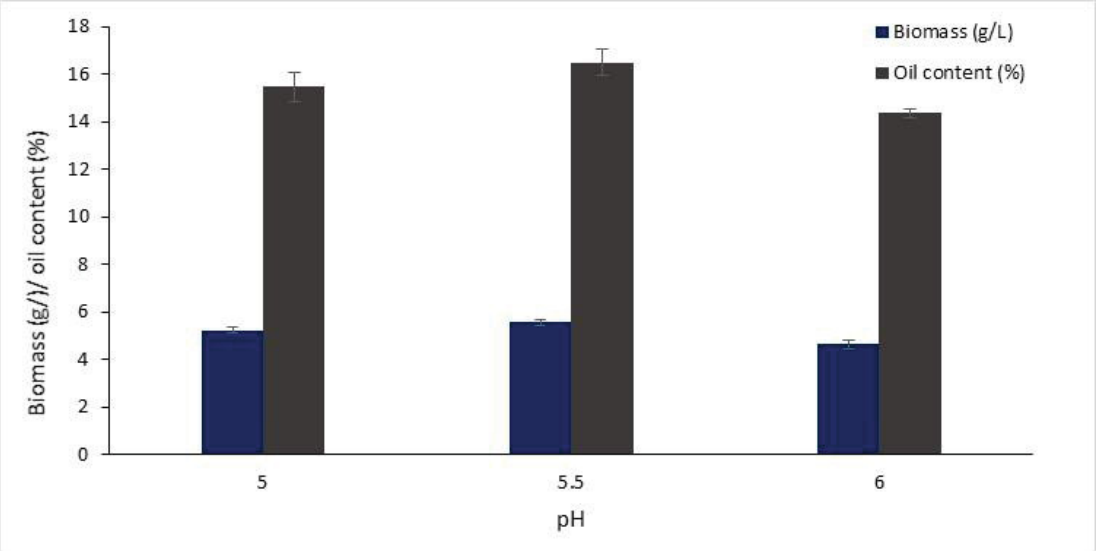
conditions of pretreatment 0.5 % (w/v) diluted sulfuric acid , this reaction was carried out at 120 °C for 60 min in an autoclave. We observed that there was greater growth and oil production of *Rhodotorula mucilaginosa* in the hydrolysate detoxified by the adsorption with activated carbon 4.5 g/L and 26 % respectively, than in hydrolysis by overliming by adding $Ca(OH)_2$ to pH 10.0 and incubating for 1 h at 30°C) 4 g/ L of biomass and 22 % oil content. In the work done by Galafassi et. al., (2012) were tested the effect of furfural, 5-HMF and acetic acid, on growth rate and lipid production of *Rhodotorula graminis*. Furfural caused a 35% reduction in growth at the lowest concentration tested, 0.75 g/L, and inhibited growth completely at 2.5 g/L. The 5-HMF was less toxic, as already reported for other species (Liu et al., 2004), allowing the growth at concentrations higher than 2.5 g/L. With acetic acid to concentrations of 3 g/L caused the inability to grow, after 90 hours of fermentation the acetic acid affects the productivity of lipids and oil content. In the presence of 83 mM acetic acid, ethanol production by Pichia stipites was strongly inhibited (Delgenes et al., 1996), and Nigam (2001) reported that the presence of acetic acid in the hydrolysate decreased the ethanol yield and productivity considerably. The inhibitory effects of weak acids have been ascribed to two mechanisms: the uncoupling hypothesis and the intracellular anion accumulation hypothesis. The undissociated form of weak acids are lipid-soluble and can diffuse across the plasma membrane and dis-sociate due to neutral cytosolic pH, subsequently decreasing the intracellular pH and leading to a decrease in cellular ATP levels (uncoupling theory), or inhibition of the activities of some cellu-lar enzymes (intracellular anion accumulation theory)(Almeida et al., 2007; Zhao et al., 2012).

However, acetic acid can be utilized by some oleaginous yeasts as a carbon source. Hu et al. (2009) reported that acetate had little effect on lipid production in *R. toruloides* and excess acetate (120 mM) improved lipid content to 68%, Chen et al. (2009) found that acetic acid increased lipid production in *T. cutaneum* and Yu et al., ((Yu et al., 2011) found that *C. curvatus* can also utilize acetate at 243 Mm. It is important to consider that the inhibitory compounds affect some microorganisms mainly to the yeasts and bacteria on the growth, for that reason, it is necessary to look for new alternatives of detoxified that are economically viable for the hydrolysates of lignocellulose residues or to realize genetic improvement of microorganisms tolerant to the inhibitory compounds.

4.4.3 OPTIMIZATION OF SOME CULTURE CONDITIONS FOR LIPID PRODUCTION

There are several factors that affect the production of lipids such as pH, in this work, we performed an assay with three pH conditions that were 5, 5.5 and 6, as we can see in FIGURE 4.3, there is no significant effect on the production of biomass at a pH of 5 and 5.5, but when the pH is 6 there is a slight decrease in the concentration of the biomass to 4.63 g/L. About oil production, the same behavior is observed that there is no significant difference of pH 5 and 5.5, and a decrease of oil production with pH 6 of 14.37%. The yeasts in general are acid-tolerant, which has made them useful for low pH food and beverage fermentations such as wine, pickles and olives.

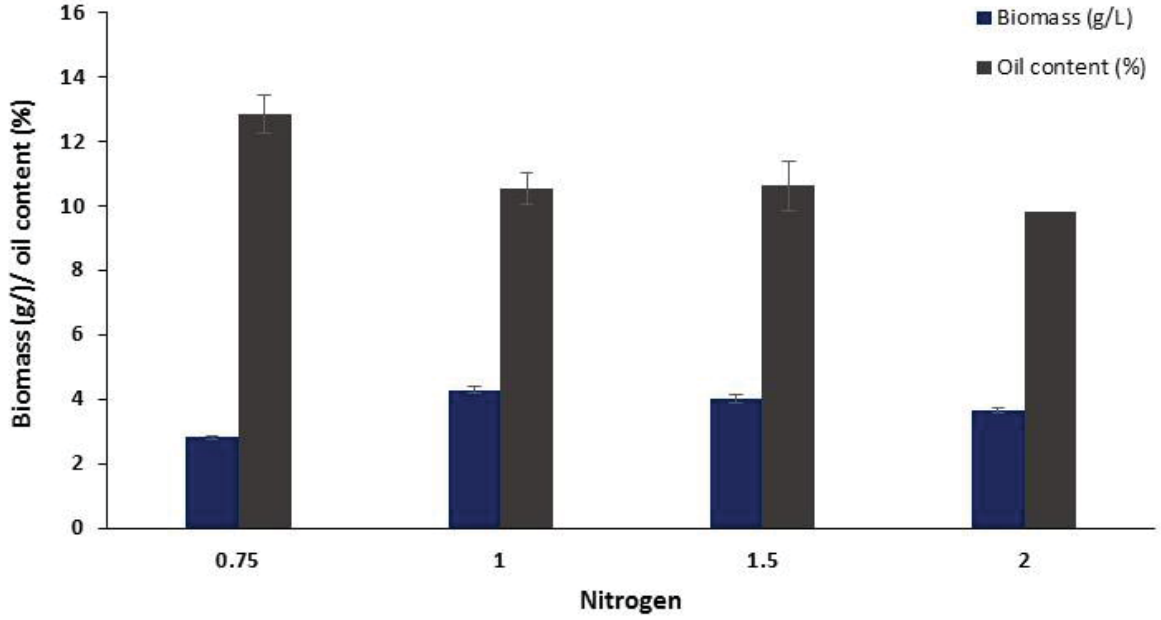
FIGURE 4.4. EFFECT OF PH ON THE PRODUCTION OF BIOMASS AND LIPID CONTENT.



Several studies have analyzed the effect of nitrogen on the production of biomass and oil content. In this work, $(NH_4)_2SO_4$ was used as a low-cost nitrogen source for lipid production, with 20 g/L of sugar from the OPEFB hydrolysate. As can be seen in FIGURE 4.4, a significant difference in oil content is seen using a concentration of 0.75 g / L with respect to 1, 1.5 and 2 g/L of ammonium sulfate, obtaining more than 12% of lipids. The presence of little or too much nitrogen affects on the concentration of biomass and lipids, in this case 0.75 g/L represents a C/N ratio of 50, the higher the carbon nitrogen ratio the greater the accumulation of lipids in the yeast. Kraisintu et al. (2010) measured cell mass and lipid production of one strain of *R. toruloides* under several conditions including varying C/N ratio, they found that the nitrogen source, glucose level and C/N ratio had significant effects. In agreement with

previous reports, increasing the C/N ratio decreased cell mass but increased lipid content; overall lipid productivity was increased only slightly at the highest C/N molar ratios of 115 and 140, with a maximum cell lipid content of 62.3% at C/N ratio of 140. Sitepu et al. (2013) cultured dozens of yeast strains in a defined low nitrogen medium, this work confirmed earlier observations that nitrogen deficiency induces higher lipid accumulation in oleaginous yeasts.

FIGURE 4.5. EFFECT THE CONCENTRATION OF NITROGEN SOURCE ON THE PRODUCTION OF BIOMASS AND LIPID CONTENT, USING AMMONIUM SULFATE.



4.4.4 BATCH FERMENTATION OF *R. mucilaginosa* IN 2 L FERMENTOR

Growth kinetics were performed at different laboratory scales, which were 2 L and 10 L. In the reactor of 10 L, were perfomed Batch and Fed Batch experiments. The growth of the yeast in a 2 L reactor is shown in FIGURE 4.5, we can see that the yeast was able to produce 5.43 g / L of biomass in 4 days with a 17.69% oil content and a productivity of 0.010 g/L/h (table 4.5) and the initial C/N ratio was 24. The low production of biomass is probably due to the inhibitory compounds present in the hydrolyzate discussed above, and to factors that influence the production of biomass, such as speed of aeration, dissolved oxygen, stirring among others.

Tampitak et al. (2015), they worked with *Rhodotorula mucilaginosa*, obtaining thus in three days 4.2 g/L of biomass and 25% of lipids with a stirring of 200 RPM using

hemicellulosic hydrolysate of OPEFB. About the consumption of sugars, we started with a initial total concentration of 19.20 g / L composed by glucose, xylose, arabinose and cellobiose. As we can see in FIGURE 4.6, the yeast consumed faster glucose than xylose, arabinose and cellobiose. The consumption lasted 5 days, because yeast has a higher preference with hexoses than with pentoses, this was also seen in the work of Liu et al., (2015), where *Rhodotorula glutinis* preferred to consume glucose first 2.27 g/L) than xylose (40 g/L) for three days. With respect to the inhibitors as shown in FIGURE 4.7, they were also consumed by the yeast before the pentoses, which is why the yeast was slow to consume these sugars. The existence of the inhibitors produces disadvantages in the xylose metabolism. In the later experiments, we will explain in more detail this assimilation of the yeasts on the compounds present in the hydrolyzate. In this growth kinetics we observed that the production of biomass and lipid content was low may be due to the operating conditions of the fermentation process, for this reason it was decided to work in a reactor of 10 L of capacity, using 5 L for to perform the growth kinetics of the *Rhodotorula mucilagionosa*.

FIGURE 4.6 THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN REATOR DE 2 L BY R. MUCILAGINOSA WITH DETOXIFIED HYDROLYSATE OPEFB

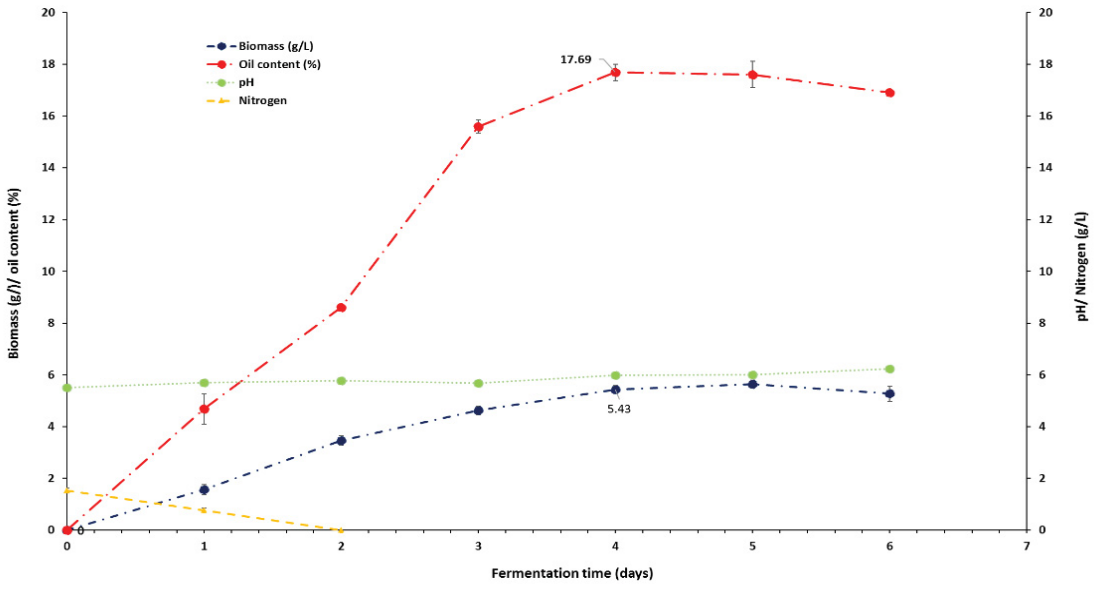


FIGURE 4.7 XYLOSE, GLUCOSE, ARABINOSE AND CELLOBIOSE CONSUMPTION.

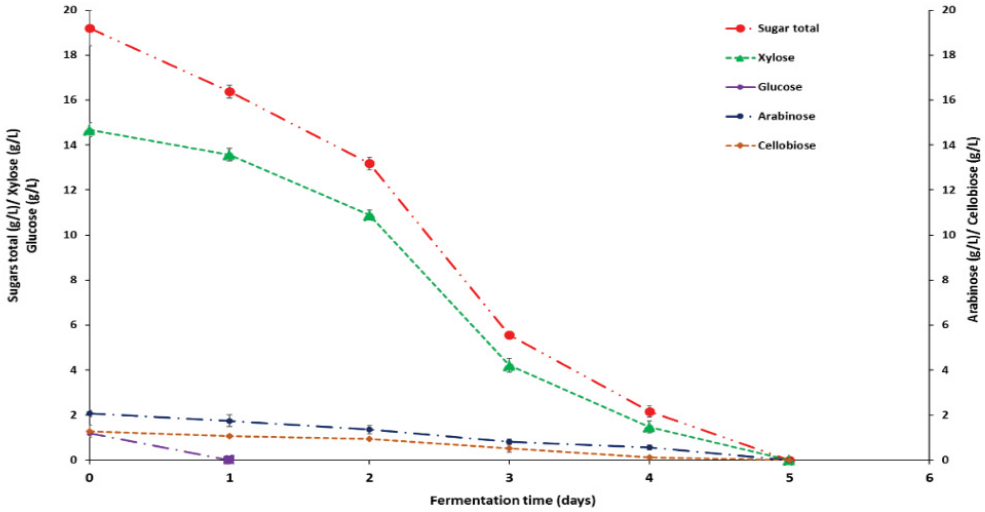
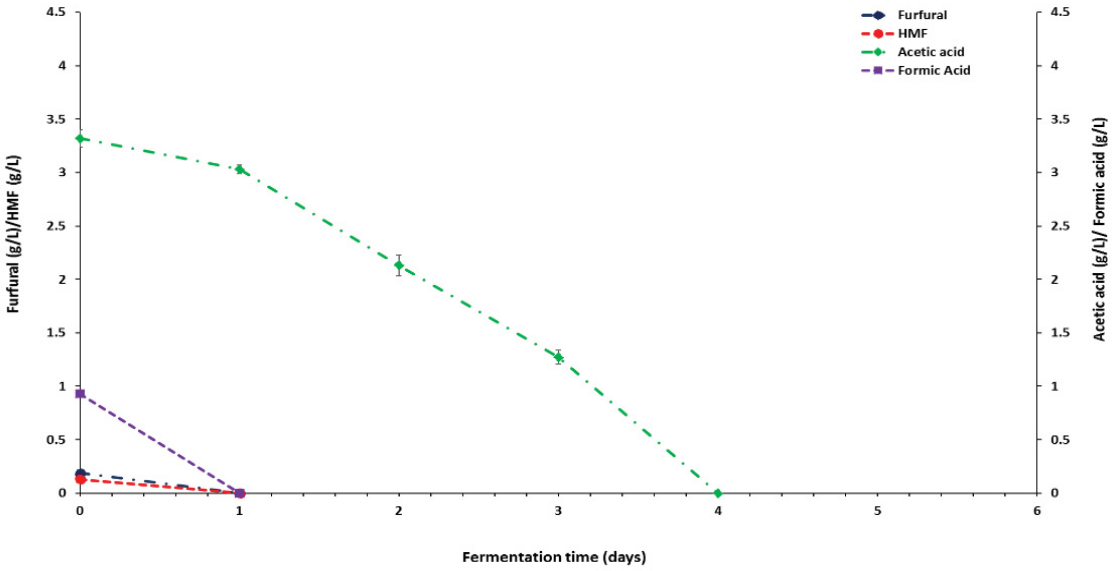


FIGURE 4.8. ACID ACETIC, HMF, FURFURAL AND ACID FORMIC CONSUMPTION BY R*hodotorula mucilaginosa.*

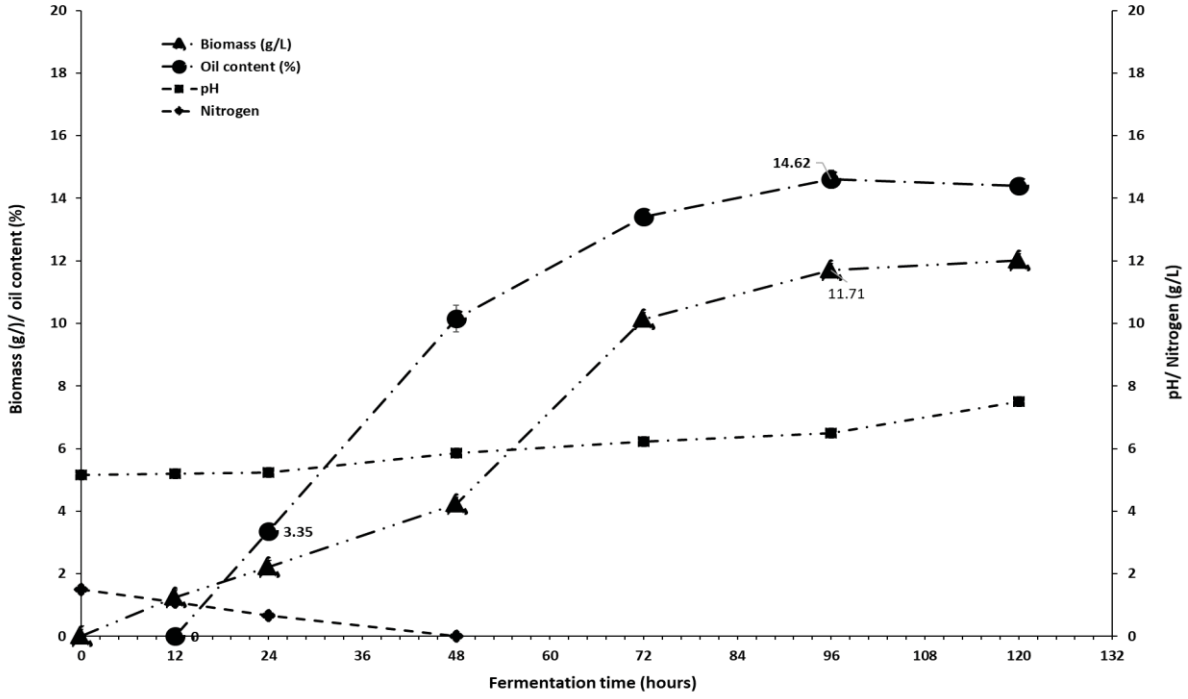


4.4.5 BATCH FERMENTATION OF *R. mucilaginosa* IN 10 L FERMENTOR

Since R. mucilaginosa showed tolerance to the inhibitors in detoxified OPEFB hydrolysate, the cell growth and lipid production in 10 L fermentor were further studied. The detoxified OPEFB hydrolysate medium with the initial sugars of 14.14 g/L was

used. The initial C/N ratio was 19.85. In spite of the existing inhibitors, the maximum biomass concentration, lipid content and lipid titer reached 11.71 g/L, 14.62 % and 1.70 g/L in 96 h, respectively. After a lag phase of 12 h, biomass increased and the specific cell growth rate was 0.0336 h-1, while the biomass increased a little more after of 48 h and the specific cell growth rate to 0.021 h-1 (Fig. 4.8).

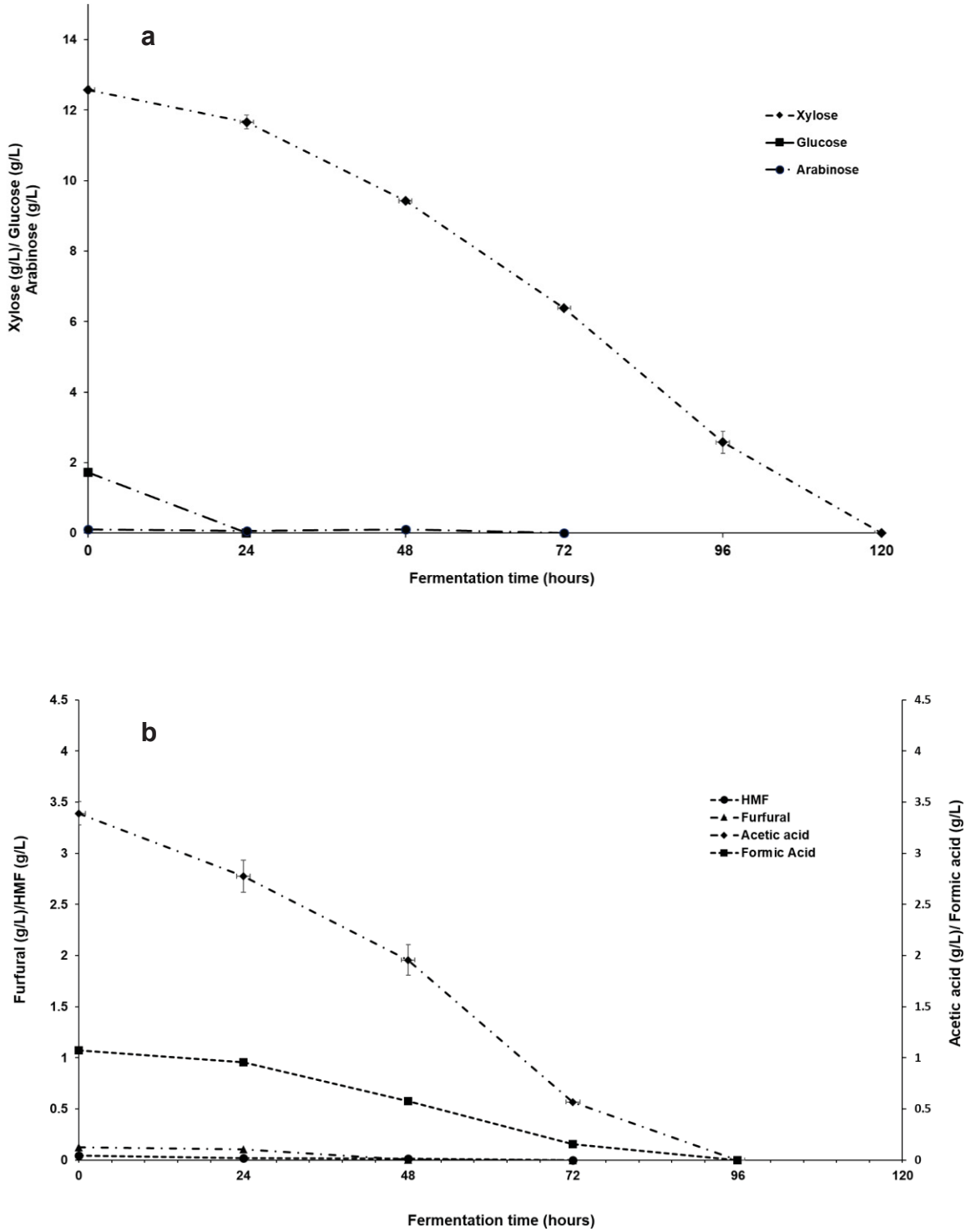
FIGURE 4.9. THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN REATOR DE 10 L BY *R. mucilaginosa* WITH DETOXIFIED HYDROLYSATE OPEFB.



The initial glucose concentration was 1.72 g / L in the hydrolysate after 24 hours of fermentation. No residue of this sugar was found (Fig. 4.9a). There is a phenomenon called repression of glucose, at least three mechanisms of repression have been reported the main mechanisms involved the phosphorylation of glucose mainly by hexokinase (Hxk2), and signals generated subsequently, resulting in transcriptional repression of genes related to respiration and assimilation of sugars which are not glucose (Colabardini et al., 2014; Kim et al., 2012; Liu et al., 2015). Due to this repression the microorganisms begin to assimilate other sugars such as arabinose, xylose and cellobiose, which are present in the hydrolysate. However as shown in Figure 5a the rate of assimilation of xylose is low. After 120 h of fermentation xylose and arabinose were consumed. There should be some other materials inhibited the

xylose metabolism by *R. mucilaginosa*. So, consumption of inhibitors in the detoxified hydrolysate was analyzed as figure 4.9b..

FIGURE 4.10. THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN REATOR DE 10 L BY *R. MUCILAGINOSA* WITH DETOXIFIED HYDROLYSATE OPEFB, A) XYLOSE, GLUCOSE AND ARABINOSE CONSUMPTION, B) ACID ACETIC, HMF, FURFURAL AND ACID FORMIC.



The concentration of furfural, acetic acid, formic acid and 5-HMF were declined faster than xylose before 96 h and all the inhibitors almost consumed up. Among these three inhibitors, furfural, HMF and formic acid were metabolized fastest than acetic acid, because they have low concentrations at the beginning of fermentation 0.28 g/L, 0.04 g/L and 1.07 g/L, respectively. The existence of the inhibitors cause disadvantages for the xylose metabolism.

Liu et al (2015) studied lipid production by Rhodotorula glutinis on hydrolysate undetoxified corncob hydrolysate, glucosa (2 g/L) was assmilted in 24 hours and xylose (40 g/L) in 72 hours. After the inhibitors were consumed to a low level, xylose concentration began to decline from 9.42 to 2.57 g/L from 48 to 120 h. Rapid lipid accumulation was from 48 to 96 h with the lipid titer increased from 0.482 to 1.712 g/L. pH was observed increasing from 5.16 to 7.5 following the inhibitors consumption. The possible reason was the acetic acid dissociation and alkaline by-products synthesis by *R. mucilaginosa* at this stage as mentioned by Liu et al. (2015) in their work, the pH of their fermentation increased with respect to time. pH decreasing was observed in some report because some acid generated during the fermentation. Wu et al., (2005) reported that pH decreased during the lipid fermentation of *Schizochytrium sp*. due to the release of organic acids such as malic acid, citric acid, pyruvic acid, and fumaric acid. However, in the fermentation broth of *R. mucilaginosa*, no distinct acid byproduct was observed. The acetic acid as substrate for lipid production by microorganisms has been reported. Christophe et al. (2012) using acetate as carbon source in the second stage to increase the lipid accumulation by C. curvatus after a significant amount of biomass obtained on glucose in the first stage, the lipid content of cells grown on acetate under nitrogen exhaustion reached a value of 47.3% dry cell weight corresponding to a lipid synthesis yield on acetate close to 0.19 (Christophe et al., 2012). However, the metabolism of furfural and 5-HMF of yeast are not clear that needs to be further studied. The results also suggested that *R. mucilaginosa* had tolerance to the inhibitors, even so under these conditions could produce lipids.

Lipid of oleaginous microorganisms accumulates in two ways, de novo lipid and ex novo lipid (Papanikolaou and Aggelis, 2011). Ex novo lipid forming based on hydrophobic substrates, while de novo lipid on hydrophilic substrates, such as glucose

and xylose. C/N ratio plays an important role in lipid production and nitrogen limitation is essential for de novo lipid accumulation (Ratledge, 2004).

The low oil production is due to the C/N ratio, which was 19.85, several authors mention that it should use at least 50, as presented in its results Liu et al. (2015) than for the best condition was to work with a C/N ratio of 75 using $(NH_4)_2SO_4$. Because of this, it was suggested to work with a Fed-batch type fermentation.

4.4.6 FED-BATCH FERMENTATION OF *R. mucilaginosa* IN 10 L FERMENTOR

Constant nitrogen feeding strategy was studied to achieve a higher production of biomass (Fig. 4.10 and Fig. 4.11). The detoxified hydrolysate medium containing 1.96 g/L glucose and 14.54 g/L xylose was used. The initial C/N ratio was 20. Concentrated detoxified hydrolysate approximately 30 g/L were repeatedly fed when the sugar concentration was below 7.5 g/L. Only the hydrolysate was detoxified at the beginning of the fermentation, and in the other feeds only the concentrated hydrolysate was adjusted to pH 5.5 with NaOH. The profiles of cell growth, substrate consumption, and lipid production are shown in FIGURE 4.10.

FIGURE 4.11. THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN A BIOREATOR OF 10 L BY *R. MUCILAGINOSA* WITH DETOXIFIED HYDROLYSATE OPEFB.

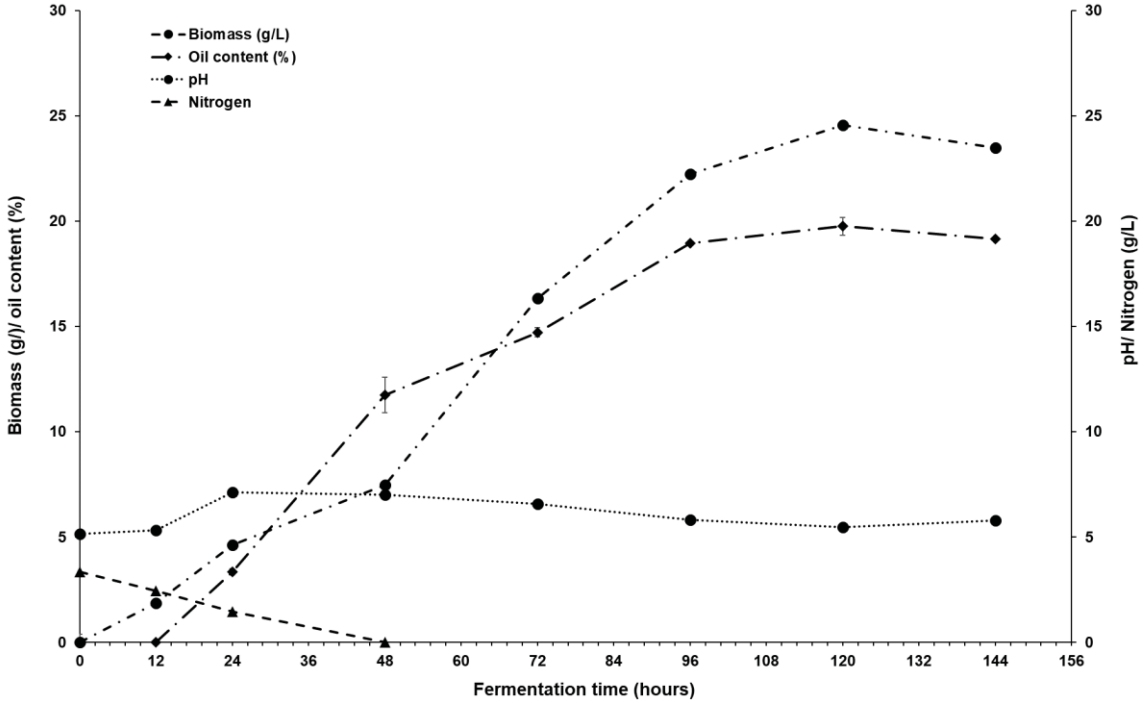
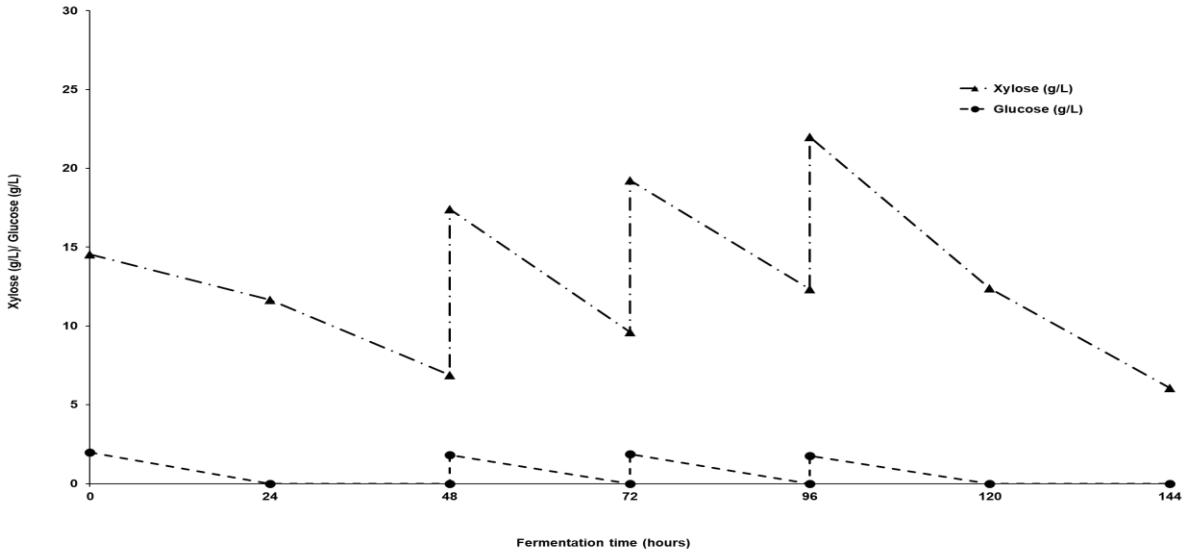


FIGURE 4.12. THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN A BIOREATOR OF 10 L BY *R. mucilaginosa* FED WITH DETOXIFIED HYDROLYSATE OPEFB, XYLOSE AND GLUCOSE CONSUMPTION.



Biomass increased fast after 48 h and 24.56 g/L biomass was obtained at 120 h, correspondingly, lipid content accumulated soon after 36 h. The final lipid content and lipid titer was 19.75 % and 4.85 g/L, respectively (table 4.5).

As we observed a fed-batch fermentation increase 2 times more the biomass production with respect to the batch fermentation that was of 11.71 to 24.56 g/L and also was due to the addition of 1 g/L of yeast extract. The oil content increased slightly from 14.62 to 19.75 in Fed-Batch, perhaps it was because the C/N ratio is still low, several researchers suggest that one should work at a C/N ratio greater than 50. Therefore it is suggested to initiate a fermentation process by fed-batch at higher sugar concentrations between 35 and 40 g/L of concentrated and detoxified hydrolysate to have a better response of the yeast in the biomass production and oil content.

TABLE 4.5. LIPID PRODUCTION UNDER DIFFERENT CONDITIONS BY *R. mucilaginosa*

Cultivation method	Biomass (g/L)	Lipid content (%)	Lipid titer (g/L)	Lipid yield (%)	Lipid productivity (g/L/h)
Batch culture by fermentor 1L	5.43	15.42	0.961	5.636	0.010
Batch culture	11.71	14.62	1.70	11.80	0.017
Fed-batch culture with 3 feed cycles	24.56	19.75	4.85	10.60	0.040

4.4.7. LIPID COMPOSITION

The amount of different fatty acids exist in the feedstock are critical to the physical properties of biodiesel because the transesterification does not change the fatty acid composition (Ramos et al., 2009). As Table 4.6 indicated, the main composition of the lipid produced with the detoxified hydrolysate was palmitic acid, stearic acid, oleic acid and linoleic acid. Oleic acid was the most abundant and linoleic acid was the least fatty acid in the case of detoxified hydrolysate as substrate. High content of unsaturated fatty acids will lead to satisfactory fuel properties at low temperatures (Zheng et al., 2012). However, excess unsaturated fatty acids play an adverse effect on the oxidative stability of biodiesel (Luque, 2010; Ramírez-Verduzco et al., 2012). The total unsaturated fatty acids of the lipid produced with detoxified hydrolysate and palm oil were 48.3% and 56.1%, which indicated that lipid from oleaginous yeast *R. mucilaginosa* has potential as a biodiesel feedstock.

TABLE 4.6. LIPID COMPOSITION OF *R. mucilaginosa.*

Lipid composition	**Value (%)**	
	Detoxified hydrolysate	**Palm oil (Saenge et al., 2011)**
Myristate acid (C14:0)	0	0
Palmitic acid (C16:0)	13	20.4
Stearic acid (C18:0)	37.5	10.3
Oleic acid (C18:1)	43.5	47.9
Linoleic acid (C18:2)	4.8	7.31
Linolenic acid (C18:3)	0	0.85
Others	0.2	0

4.5. CONCLUSIONS

In this work we conclude that by detoxification with active carbon we were able to remove 92.63 % of furfurals, 91.52 % of HMF, 33.48 % of acetic acid and 14.48 % of formic acid. The importance of eliminating them is that they influence the growth and production of lipids of *Rhodotorula mucilaginosa*, mainly high amounts of acetic acid. It was possible to produce 24.56 g/L of biomass and 19.76% of lipids content in 5 days by a fermentation bioprocess in Fed-batch using *Rhodotorula mucilaginosa*, which indicates that using hydrolysis of the OPEFB hemicellulose could be an alternative for the production of microbial oil. The fatty acids present in the *Rhodotorula mucilaginosa* oil using detoxified hydrolysate are mainly composed of palmitic acid, stearic acid, oleic acid and linoleic acid, which indicated that had potential as feedstock for production of biodiesel.

4.6 SUGGESTIONS

It is recommended in future experiments to make a fed-bacth with a concentration of 40 g/L of dehydrated hydrolysate sugars and to reduce feeding times, to obtain a greater production of microbial oil with *Rhodotorula mucilaginosa*. In these operating conditions (250 RPM, 1.5 vvm, 10% inoculum) experiments can be performed with *Rhodotorula glutinis* in a 10 L reactor type batch and fed batch, although it did not present good production of biomass in flask, perhaps in a reactor of greater capacity increase productivity, as seen in this study.

5. CHAPTER III: DEVELOPMENT OF A PROCESS TO PRODUCE MICROBIAL OIL USING SUGAR CANE MOLASSES

ABSTRACT

Sugar cane molasses, a by-product of sugar industry, was used as the sole carbon source for production of lipids by oleaginous red yeast *Rhodotorula glutinis* PPGEBB115. The addition of ammonium sulfate whit yeast extract as nitrogen sources and Tween 20 as a surfactant, increased the accumulation of lipids. Among the factors investigated using response surface methodology, the C/N ratio contributed a significant effect on biomass production and lipid content. The synergic effects of nitrogen concentration with sugar cane molasses concentration and time were observed in the accumulation of lipids. The optimum condition for biomass production was molasses concentration of 4 % and 4 g/L of ammonium sulfate, while that for lipid content was molasses concentration of 6 % and 2 g/L of ammonium sulfate, in a flask. The production of lipids was further improved in a stirred tank bioreactor with pH at 5.5, aeration rate at 1.5 vvm and 1.5 g/L of Tween 20. In fed-batch fermentation, a highest biomass production of 27.95 g/L with a cellular lipid content of 60.94 % and 3.40 g/L/day of lipid productivity were obtained. The main fatty acids presents in *R. glutinis* oil were oleic acid 63.59 %, palmitic acid 20.81 %, stearic acid 6.68 %, linoleic acid 5.42 %. The results obtained with *Rhodotorula glutinis* have shown the favorable properties for being used as feedstock in the production of biodiesel.

5.1 INTRODUCTION

The main global problem of economic, social, environmental and human survival is climate change (IPCC, n.d.) . Climate change is a climatic transformation caused by emissions of greenhouse gases (GHG) derived from human activities from the generation of the Industrial revolution. The Intergovernmental Panel on Climate Change (IPCC) concluded that this phenomenon began more than two centuries ago and humans contribute every day to increase it. One of the alternatives to reducing greenhouse gas emissions (GHG) is the diversification of the energy matrix through the use of biofuels such as biodiesel and bioethanol (Soccol et al., 2017) biodiesel is

a renewable, safe and non-toxic biofuel that can be obtained from edible and non-edible oleaginous feedstocks (Vyas et al., 2010).

The conversion of oleaginous raw materials into biodiesel is carried out through the homogeneously or heterogeneously catalyzed transesterification of largely branched triglycerides (TG) into smaller, straight-chain esters (Pinzi et al., 2011). The high cost of feedstocks to produce biodiesel is the a major obstacle in the commercialization of the product (Birla et al., 2012). On the other hand, if the raw materials are from agricultural crops, it is often asserted that this type of biodiesel can contribute to rising food prices (Deng et al., 2012). Adoption of animal fats used frying oils and waste oils from restaurants as feedstock is a good strategy to reduce the cost. However, these limited resources cannot meet the increasing needs for clean renewable fuels (Huang et al., 2009). In this context, it is considered relevant and necessary to explore new sources of obtention of oil as feedstock for producing biodiesel. Microbial oil production by oleaginous yeasts and moulds could be used as feedstock for biodiesel due to its structure and composition is like that of common vegetable oil (Beopoulos et al., 2011; Meng et al., 2009; Papanikolaou and Aggelis, 2002; Wang et al., 2013). There are a wide variety of microorganisms belonging to the genera algae, bacteria, yeasts and fungi have the ability to accumulate lipids under specific cultivation conditions. Among them, the use of oleaginous yeast has several advantages due to the rapid growth rate and the high oil content compared to algae (Li et al., 2008; Subramaniam and Dufreche, 2010), especially for *Rhodotorula glutinis* which was reported to accumulate around 70% (w/w) lipid in dry weight under nitrogen-limited conditions (Xue et al., 2008). The accumulation of lipids in oil-producing microorganisms basically occurs under conditions of limited nitrogen and in the presence of high sugar content (Economou et al., 2010). During the growth phase, nitrogen is needed for the synthesis of proteins and nucleic acids, while the carbon source is used for the energy and anabolic processes that produce carbohydrates, lipids, nucleic acids and proteins (Leiva-Candia et al., 2014). Since microbial lipids can be produced using industrial waste and lignocellulosic material as substrate, showing good characteristics such as short and low life-threatening weather conditions (Amaretti et al., 2010). Although the microbial oil has some advantages compared to plant oil and animal-derived oil, the current problem to be solved is the reduction of the cost to improve its economic competition. Therefore, exploring ways to reduce the high cost of biodiesel is of much interest in recent research. An alternative approach is the

use of inexpensive feedstock, such as waste or residual materials for lipid production by oleaginous microorganisms (Papanikolaou and Aggelis, 2002).

Sugarcane molasses is a by-product generated by the sugar industry that can be harnessed and converted with high valorization, is comparative low in price compared to other sources of sugar and rich in various nutrients besides sucrose (Veana et al., 2014; Wang and Ren, 2014). Brazil, which is the main producer of sugar cane worldwide with a production of more than 736 million tonnes (FAOSTAT (2017), n.d.). Molasses is the final effluent obtained in the preparation of sugar by repeated crystallization; it is the residual syrup from which no crystalline sucrose can be obtained by simple means. The yield of molasses is approximately 3.0 percent per tonne of cane but it is influenced by a number of factors and may vary within a wide range (2.2 to 3.7 percent) (Paturau, 2011). The chemical composition of molasses is very varied mainly containing approximately 35% sucrose, reducing sugars (9% fructose and 7% glucose) and trace elements, are the principal compounds. Thus, sugar cane molasses is an attractive substrate for microbial oil production by *R. glutinis.*

5.2 OBJECTIVES

Develop a process to produce microbial oil from sugar cane molasses using Rhodotorula glutinis.

5.2.1 SPECIFIC OBJECTIVES

5.2.1.1 Optimize a process to produce microbial oil from *Rhodotorula glutinis* using sugar cane molasses

5.2.1.2 Optimize a process to produce microbial oil in a 10-liter reactor from *Rhodotorula glutinis* using sugar cane molasses.

5.3 MATERIALS AND METHODS

5.3.1 MICROORGANISM AND MEDIA

Rhodotorula glutinis PPGEBB115, was obtained from the isolation of soil and was used to produce lipids. It was maintained on a yeast and malt extract agar slant (g/L, glucose 10, peptone 5, yeast extract 3, malt extract 3 and agar 15) at 4° C until used. A plate culture was incubated at 30° C for 24 h. The cell was then transferred to 125 mL flask containing 25 mL of culture medium (g/L, glucose 10, peptone 5, yeast extract 3 and malt extract 3). The flasks were incubated at 30° C and 120 rpm for 24 h for seed culture. The carbon source used in liquid medium was sugar cane molasses, composed of various sugars, minerals and others (TABLE 5.1).

TABLE 5.1 COMPOSITION OF SUGAR CANE MOLASSES

Compositions	Quantidade
Calcium	11.45 g/Kg
Ash	8.7 % (w/v)
Cobalt	0.001 g/Kg
Copper	0.042 g/Kg
Dextran	6.073 g/Kg
Iron	0.071 g/Kg
Total phosphorus	0.873 g/Kg
Fructose	7.9 % (w/w)
Glucose	6.98 % (w/w)
Magnesium	3.991 g/Kg
Manganese	0.291 g/Kg
Potassium	25.57 g/Kg
Sodium	0.119 g/Kg
Sulphite	2.114 g/Kg
Zinc	0.062 g/Kg
Water	5.48

5.3.2 OPTIMIZATION THROUGH RESPONSE SURFACE METHODOLOGY (RSM)

A Rotational Central Composite Design (DCCR) with three variables including sugar cane molasses concentration, nitrogen concentration and time; at three levels was followed to determine the response pattern and to determine the synergy of variables. According to this design, 17 runs were conducted containing three replications at the central point and two axial points for estimating the purely experimental uncertainty variance (table 5.2). The experiments were conducted in 250

mL Erlenmeyer flasks. The sugar cane molasses was diluted to obtain 4, 6 and 8 % of molasses concentration. Ammonium sulfate as a nitrogen source was added to obtain 1, 2 and 3 g/L. The pH was adjusted to 5.5 prior to use as a molasses based medium. The seed culture was 10% (v/v) inoculated to 90mL molasses based medium and incubated at 28 °C and 120 rpm for 96, 144 and 192 h depending on the treatment. The relationship of the variables was determined by fitting a second order polynomial equation to data obtained from the 17 runs. The response surface analysis was based on the multiple linear regression which considering the main, quadratic and interaction effects, in accord with the following equation:

$$Y = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j represent the variables or parameters, β_o is the offset term, β_i is the linear effect, β_{ij} is the first order interaction effect and β_{ii} is the squared effect. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Response surface plots were developed to indicate an optimum condition using the fitted quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

TABLE 5.2 ROTATIONAL CENTRAL COMPOSITE DESIGN (DCCR) WITH THREE VARIABLES

Coding and levels of experiment factors						
Factor	**Symbol**	**Code level**				
		- 1.68	**-1**	**0**	**1**	**+ 1.68**
Sugar concentration (%, w/w)	X_1	2.64	4	6	8	9.63
Nitrogen concentration (g/L)	X_2	0.32	1	2	3	3.68
Fermentation time (h)	X_3	63.12	96	144	192	224.64

5.3.3 EFFECT OF SURFACTANT ON OIL PRODUCTION

With the optimized medium, it was evaluated the effect of the surfactant (Tween 20) on the production of oil using concentrations of 0.5, 1, 1.5 and 2 g/L. The seed culture was 10% (v/v) inoculated to 90 mL molasses based medium and incubated at 28 °C and 120 rpm for 6 days. Biomass production and lipid content were determined. The experiment was performed in triplicate.

5.3.4 BATCH FERMENTATION

The batch cultures were carried out in a 10 L stirred tank bioreactor contained 4 L optimized medium from RSM. The culture temperature was 28° C, the initial pH was 5.5 and was not controlled during fermentation, the aeration rate was of 1.5 vvm, 250 RPM and 7 days of fermentation. The time courses of biomass, lipid yield per liter and concentration of sugar consumed were determined.

5.3.5 FED-BATCH FERMENTATION

In the fed-batch fermentation, the start-up of the process was the same as with the batch operation. The feeding started at 48 hours and every 48 hours 3% of sugar cane molasses was fed to maintain the optimum level obtained from RSM, also added 1.5 g/L of tween 20. The pH of the medium was not controlled. Biomass time cycles, lipid yield per liter and the concentration of sugars were determined.

5.3.6 ANALYTICAL METHODS

Biomass was harvested by centrifugation at 3500rpm for 15 min. The pellets were then washed with distilled water twice and dried at 60° C in hot air oven to get constant weight. Extraction of lipids from biomass was performed according to the modified procedure of Bligh and Dyer (Bligh and Dyer., 1959). Lipids were extracted

with a mixture of chloroform: methanol (2:1, v/v) for 1 h. The extracted lipids were centrifuged to obtain a clear supernatant and the solvent was removed. The method for fatty acid methyl esters (FAME) production from extracted lipids involved hydrolysis of the lipids and followed by esterification (Jham et al., 1982). The fatty acid composition in the FAME were analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32mmI.D., 0.25_mfilm thickness) and flame ionization detector. Operating condition were as follows: inlet temperature 290 ◦C, oven temperature initial 210 ◦C hold 12 min ramp to 250 ◦C at 20◦C/min, hold 8min and detector temperature was 300 ◦C. Fatty acids were qualified by comparing their retention times with those of standard ones. The sugar concentration was determined following the method of DNS. The statistical significance of the results was evaluated by one-way ANOVA (analytical of variance) using the Statsoft statistica version 7 software.

5.3.7 CALCULATION OF BIODIESEL PROPERTIES

$$AU=\Sigma \times NCi \quad (2)$$

where N is the number of carbon–carbon double bonds of unsaturated fatty acids and Ci is the concentration (mass fraction) of the component. Each property was calculated using Eqs. (2) – (7) (Hoekman et al., 2012).

$$\text{Viscosity} = -0.6316AU + 5.2065 \quad (3)$$

$$\text{Specific gravity} = 0.0055AU + 0.8726 \quad (4)$$

$$\text{Cloud point} = -13.356AU + 19.994 \quad (5)$$

$$\text{Cetane number} = -6.6684AU + 62.876 \quad (6)$$

$$\text{Iodine number} = 74.373AU + 12.71 \quad (7)$$

$$HHV = 1.7601AU + 38.534 \quad (8)$$

5.4 RESULTS AND DISCUSSION

5.4.1 MEDIUM OPTIMIZATION THROUGH RSM

It is known that lipids production requires a medium with an excess of the carbon source and limited other nutrients, usually nitrogen. Thus, the oleaginous potential is critically affected by the ratio of carbon and nitrogen sources (C/N ratio) of the culture (Saenge et al., 2011).

Sugar cane molasses is a residue that is composed of several compounds in addition to the sugars, we find several minerals and even ashes in its composition, so it is important to conduct a study to know how much is the capacity for assimilation of yeast for its growth. Therefore, it is important to determine the optimal condition for both biomass and lipids accumulation. In this study, three main factors, namely molasses concentration, nitrogen concentration and fermentation time, were selected for the optimization of biomass and lipid content. The Rotational Central Composite Design (DCCR) experiment design led to a total 17 sets of experiments. The low,

middle, and high levels of each variable and the experimental design, respective experimental results are given in TABLE 5.3.

TABLE 5.3 EXPERIMENTAL RANGE AND LEVELS OF THE THREE INDEPENDENT VARIABLES USED IN RSM IN TERMS OF CODED AND ACTUAL FACTORS AND EXPERIMENTAL DATA FOR THE THREE-FACTOR WITH THREE-LEVEL RESPONSE SURFACE ANALYSIS.

Trial	Independent variable			Dependent variable		
	Molasses (%)	Nitrogen (g/L)	Time (days)	Biomass (g/L)	Oil content (%)	Oil production (g/L)
1	-1 (4)	-1(1)	-1(4)	2.3	22.04	0.506
2	-1 (4)	-1(1)	1(8)	5.19	26.2	1.359
3	-1(4)	1(2)	-1(4)	4.85	24.8	1.202
4	-1(4)	1(3)	1(8)	7.54	28.95	2.182
5	1(8)	-1(1)	-1(4)	2.42	23.08	0.558
6	1(8)	-1(1)	1(8)	6.3	26.38	1.661
7	1(8)	1(3)	-1(4)	3.6	14.36	0.516
8	1(8)	1(3)	1(8)	3.44	25.01	0.860
9	-1.68(2.64)	0(2)	0(6)	5.36	18.19	0.974
10	1.68(9.36)	0(2)	0(6)	2.3	16.56	0.380
11	0(6)	-1.68(0.32)	0(6)	2.36	20.8	0.491
12	0(6)	1.68(3.68)	0(6)	5.01	19.62	0.983
13	0(6)	0(2)	-1.68(2.63)	4.18	20.22	0.845
14	0(6)	0(2)	1.68(9.36)	7.76	31.11	241.4
15	0(6)	0(2)	0(6)	5.18	27.45	0.142
16	0(6)	0(2)	0(6)	5.97	26.87	1.604
17	0(6)	0(2)	0(6)	5.12	29.91	1.531

The experiments were conducted in 250mL Erlenmeyer flasks. The seed culture was 10% (v/v) inoculated to 90mL molasses based medium and incubated at 28° C and 200rpm for 72 h. The results obtained by Rotational Central Composite Design (DCCR) were analyzed by ANOVA (TABLE 5.4 and 5.5).

TABLE 5.4 REGRESSION OF COEFFICIENTS AND ANALYSIS OF VARIANCE OF THE SECOND ORDER POLYNOMIAL FOR BIOMASS.

Coefficient	Biomass Y_1 (Regression coefficient)	Sum of squares	P-value
β_0	-12.011		
Linear			
X_1	2.129	9.07398	0.0028
X_2	8.686	6.24906	0.00064
X_3	0.464	12.08569	0.3607
Interaction			
X_1X_2	-0.559	7.62296	0.0004
X_1X_3	0.0088	0.00907	0.8304
X_2X_3	-0.411	4.13028	0.0021
Quadratic			
X^2_1	-0.124	2.69124	0.0064
X^2_2	-0.533	3.13759	0.0043
X^2_3	0.0657	0.76481	0.081
Pure error		1.285	
Residual	0.1836		
Total	48.063		
R^2 of model	0.973		

NOTA: Significant effect ($p<0.05$)

TABLE 5.5 REGRESSION OF COEFFICIENTS AND ANALYSIS OF VARIANCE OF THE SECOND ORDER POLYNOMIAL FOR LIPID CONTENT.

Coefficient	Lipid content Y_2 (Regression coefficient)	Sum of squares	P-value
β_0	-13.435		
Linear			
X_1	10.111	9.07398	0.001
X_2	5.78	6.24906	0.162
X_3	0.818	12.08569	0.676
Interaction			
X_1X_2	-0.6166	7.62296	0.115
X_1X_3	0.0233	0.00907	0.885
X_2X_3	-0.411	4.13028	0.028
Quadratic			
X^2_1	-0.784	2.69124	0.0004
X^2_2	-2.186	3.13759	0.0036
X^2_3	-0.098	0.76481	0.464
Pure error		1.285	
Residual	2.871		
Total	345.89		
R^2 of model	0.941		

NOTA: Significant effect ($p<0.05$)

The second order regression equations for biomass (Y_1) and lipid content (Y_2) and as a function of molasses concentration (X_1), Nitrogen concentration (X_2) and time (X_3) are given as follows:

$$\text{Biomass concentration } (Y_1) = -12.011 + 2.129X_1 + 8.687\,X_2 + 0.464X_3 - 0.124{*}X^2_1 - 0.533{*}X^2_2 + 0.065\,X^2_3 - 0.559X_1X_2 + 0.0088X_1X_3 - 0.411X_2X_3 \quad (9)$$

$$\text{Lipid content } (Y_2) = -13.436+10.11X_1+5.780X_2+0.818X_3-0.784X^2_1-2.186X^2_2-0.098X^2_3-0.617X_1X_2+ 0.0233X_1X_3+0.94X_2X_3 \quad (10)$$

The models fitted satisfactorily with the experimental data as indicated by their good of fit expressed by R^2 and P values (Table 5.4 and 5.5). The R^2 values of the models for Y_1, Y_2, were 0.97 and 0.94, respectively. This indicated that up to 94 –98 % of the variations in biomass and lipid content can be explained by these equations. Further statistical analysis in Table 5.4 showed that molasses concentration (X_1) and nitrogen concentration (X_2) had a significant effect on all the responses ($P < 0.05$). The interaction terms of X_1X_2 and X_2X_3 were found significant for biomass. With respect in the table 5.5 showed that molasses concentration had a significant effect on all the responses ($P < 0.05$) but not nitrogen concentration, X_2X_3 was found significant for lipid content. Among three variables, the effect of time in the range examined was found not significant for all the responses, but in the interactions there is significant difference.

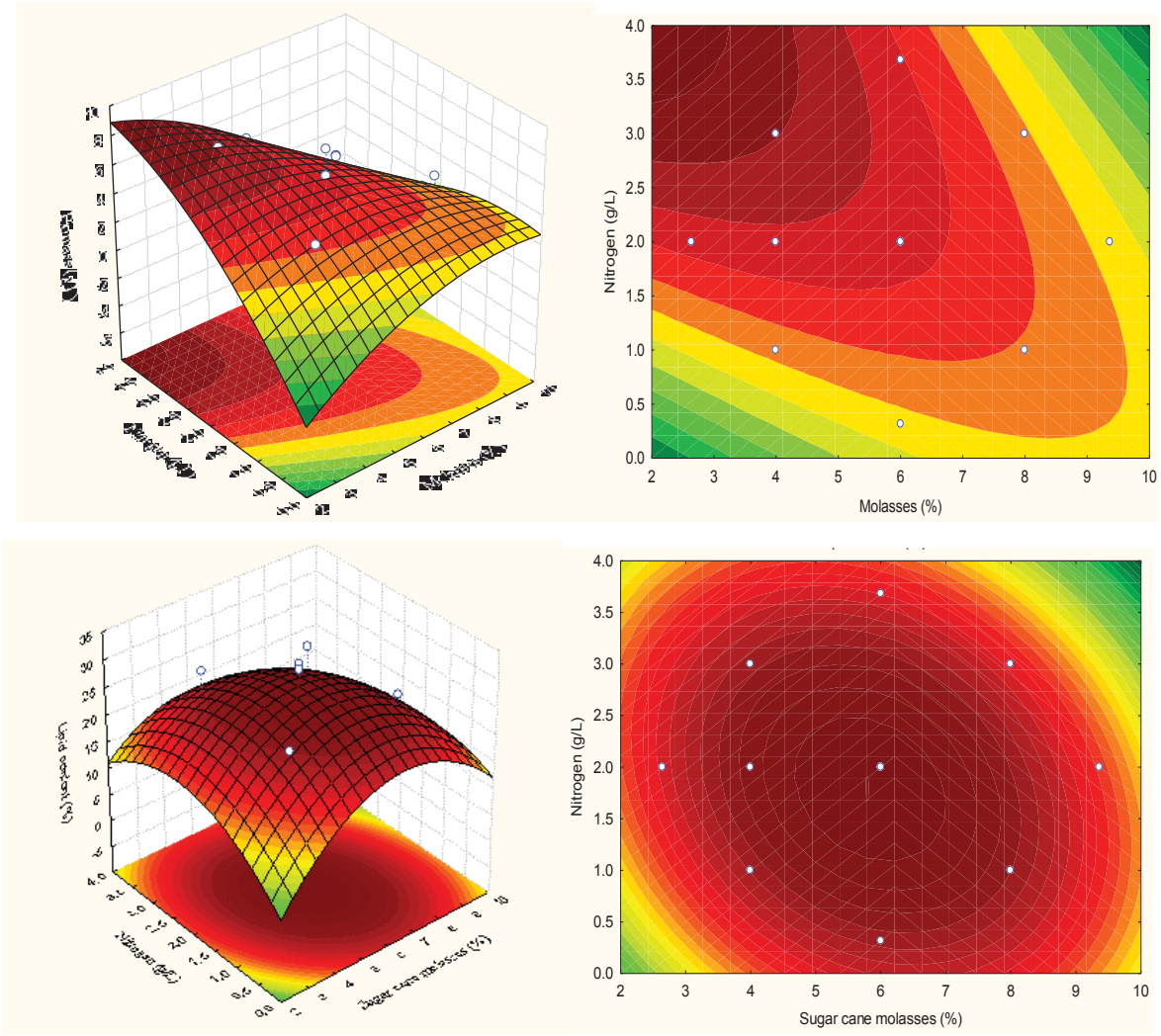
Thus, the mathematical model was simplified, by using term of time (X_3) at optimum value of 6 days. Then, in further regression analysis, the main, quadratic, and interaction effects of X_1 and X_2 were maintained for biomass and for oil content the main, quadratic, and interaction effects of X_1, X_2 and X_3 were maintained. The second order regression equation for biomass (Y_1) and lipid content (Y_2) as a function of molasses concentration (X_1) and nitrogen (X_2) generated regression relationship as given in Eqs. (11) and (12).

$$\text{Biomass} = - 6.8642 + 2.129X_1 + 8.687\ X_2 - 0.124*X_1^2 - 0.533*X_2^2 – 0.559X_1X^2 - 0.411X_2X_3 \quad (11)$$

$$\text{Lipid content} = -12.063+10.11X_1 + 5.780X_2-0.784X^2{}_1- 2.186X^2{}_2-0.617X_1X_2 + 0.023X_1X_3 + 0.94X_2X_3 \quad (12)$$

The regression models were employed to develop response surface plots. The plots of biomass and lipid content illustrated the effects of molasses concentration and nitrogen concentration. The relationship that was obtained between molasses concentration and nitrogen concentration were illustrated as three-dimensional response surfaces and two-dimensional contour plots generated by the models as shown in FIGURE 5.1

FIGURE 5.1 - RESPONSE SURFACE PLOTS AND CONTOUR PLOTS FOR THE EFFECT OF MOLASSES CONCENTRATION (X_1) AND NITROGEN CONCENTRATION (X_2) ON BIOMASS (A) AND LIPID CONTENT (B), WHEN TIME WAS FIXED AT 6 DAYS.



Based on the response surface plots, the interaction between two variables and their optimum levels can be easily understood and located. The optimum values of these factors were obtained by solving the regression Eqs. (4) and (5).

At the range tested, the optimal levels of molasses concentration and nitrogen concentrations for biomass production were 4 % and 4 g/L, respectively, at which the maximum biomass of 7.08 g/L would be obtained (FIGURE 5.1A), under this condition, the lipid content was 16.92 % (FIGURE 5.1 B). At a molasses concentration, higher than 9.32 % cell growth was inhibited. The possible reason for this could be that a high concentration of molasses could result in a high osmotic pressure and consequently inhibit the metabolic activity of the cell (Saenge et al., 2011). The optimal levels of molasses concentration and nitrogen concentrations for lipids content were 6

% and 2 g/L, respectively, at which the maximum lipids content of 28.02 % would be obtained (FIGURE. 5.1 B). This condition was then experimentally tested. The responses obtained from the predicted conditions compared with the actual values are shown in Table 5.6. The actual values of the biomass and lipid content obtained using the above optimum condition were 5.41 g/L and 27.92 %, respectively. To further increase the biomass production and lipid content, batch fermentation was carried out in a 10L stirred tank bioreactor. The effects of the culture conditions, including pH and aeration rate, were determined.

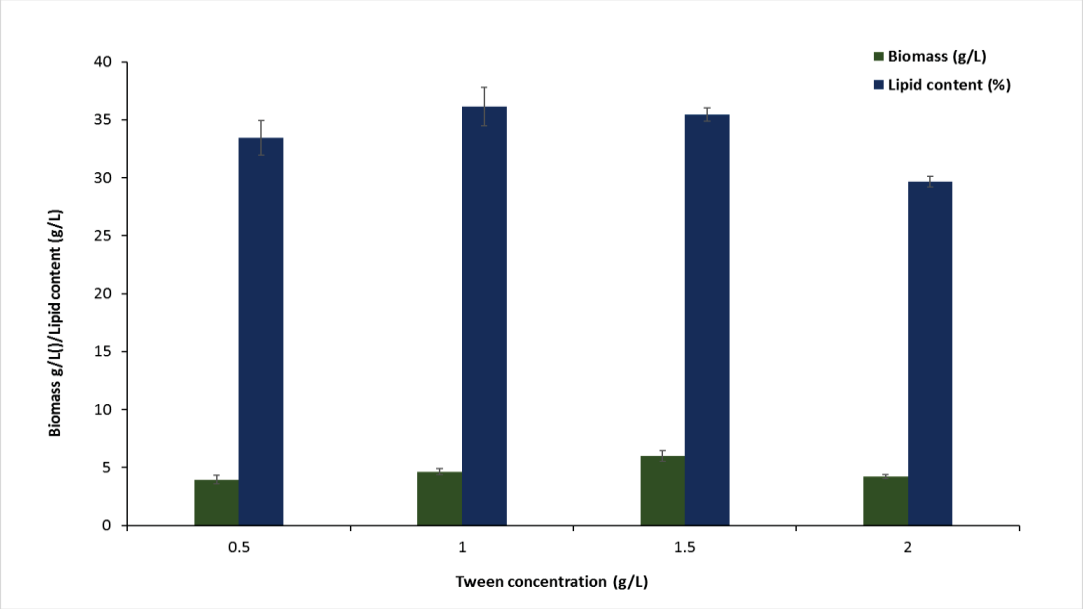
TABLE 5.6. PREDICTED AND OBSERVED VALUES FOR THE DEPENDENT VARIABLES AT OPTIMUM CONDITION FOR LIPID CONTENT.

Response	Predicted value	Actual values
Biomass (g/L)	5.06	5.41
Lipid content (%)	28.01	27.92

5.4.2 EFFECT OF SURFACTANT ON OIL PRODUCTION

Some agents, such as detergent additives, oil and surfactants have been known to increase lipids productivity (Kim et al., 2006). The mechanism of this stimulation is not completely clear, but the agents appear to cause different alterations in membrane fluidity (Kruszewska et al.,1990). In this study, the experiments were conducted in 250 mL Erlenmeyer flasks. The sugar cane molasses was diluted to obtain 6% molasses concentration. Ammonium sulfate was then added as a low-cost nitrogen source to obtain a C/N ratio of 22. The surfactant that was used was Tween 20, were tested in concentrations of 0.5 g/L, 1.0 g/L, 1.5 g/L and 2 g/L, for the enhancement of lipids production by R. glutinis PPGEBB115. The seed culture was 10% (v/v) inoculated to 90mL sugar cane molasses based medium and incubated at 28° C and 120 rpm for 6 days. The results are shown in the FIGURE 5.2.

FIGURE 5.2 - EFFECT OF TWEEN 20 ON BIOMASS AND LIPIDS PRODUCTION.



Among the four investigated concentrations, using 1.5 of Tween 20 was found to be most effective in increasing both lipid content and biomass production. It gave the highest amounts of biomass and lipid content (6.05 g/L and 35.47%). The concentrations of 1 and 1.5 had no significant difference with respect to the oil content of 36.14 and 35.47% but with biomass production if there was significant difference 4.65 g/L and 6.05 g/L, respectively. This high production and consumption of molasses, could be because the surfactant emulsified hydrocarbon-based compounds, improves the assimilation of nutrients, so that the microorganisms can then more efficiently digest (Bodour et al., 2003). These results are similar with the work done by Saenge et al. (2011) using tween 20 in a medium rich with glycerol using *Rhodotorula glutinis*. It was reported that Tween 20 added into the medium stimulated the growth, improved the growth rate and hence enabled the excretion of the product out of cells (Dalmau et al., 2000). Previous research has found that Tween 20 as a carbon source was suitable for lipids production but it was not good for cell growth (Li et al., 2006). In this study, the addition of Tween 20 enhanced both cell growth and lipids accumulation.

5.4.3 BATCH FERMENTATION

A batch fermentation process was carried out in a 10 L stirred tank bioreactor. In 5 L of medium was placed with sugar cane molasses and different parameters were evaluated every 24 hours for 7 days. In Figure 5.3 and 5.4 it can be observed that 9.58 g / L of biomass, 53% of lipid content and a productivity of 0.042 g/L.h were obtained. The C/N ratio was 26, although a low ratio resulted in a higher oil content of 53%, that is to say, a 70% increase over the optimized medium in a shake flask culture. We also observed that their productivity was low due to the low production of biomass. At 6 days, we observed that even the yeast continued to grow, but the oil content began to decrease from 53.44% to 49.19%. This may be because the yeasts were using lipids as carbon source because they had no more sugars present in the medium. Due to the low yield, it was decided to work on a Fed Batch process with the objective of increasing productivity in the production of microbial oil.

FIGURE 5.3 - THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN A 10 L FERMENTOR BY R. GLUTINIS WITH SUGAR CANE MOLASSES

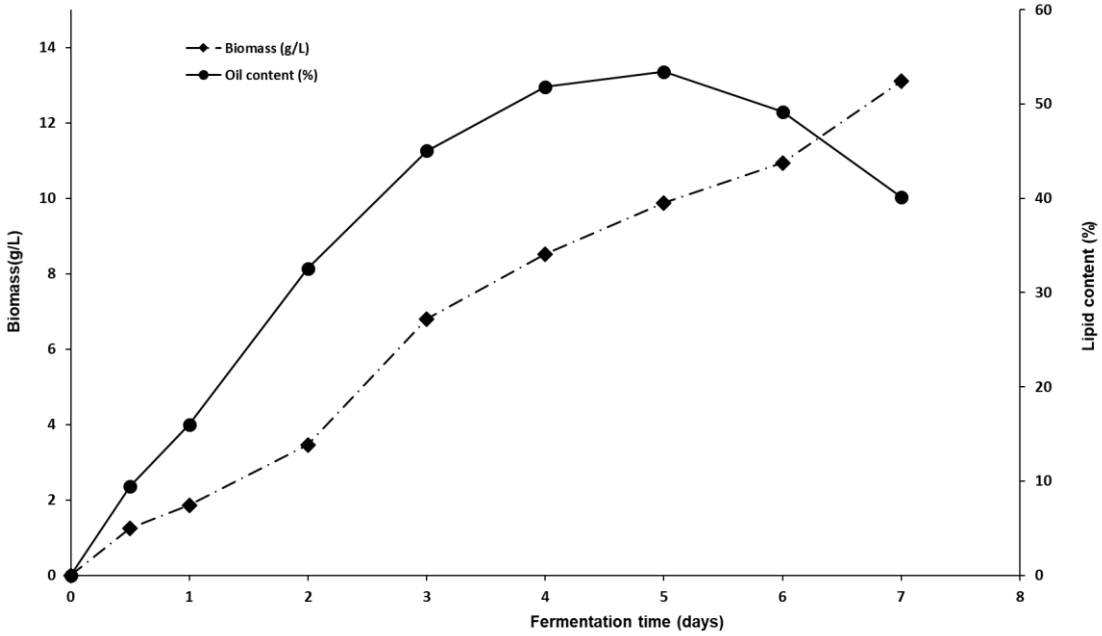
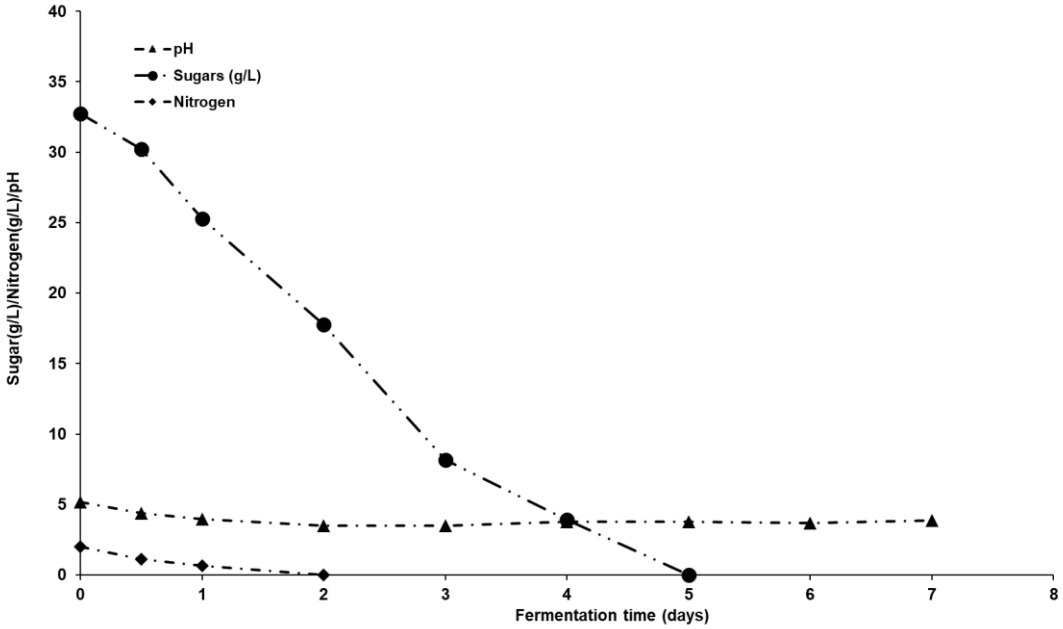


FIGURE 5.4 - THE TIME COURSE SUGARS AND NITROGEN CONSUMPTION, PH NOT CONTROLLED IN A 10 L FERMENTOR WITH R. GLUTINIS WITH SUGAR CANE MOLASSES.



5.4.4 FED BATCH FERMENTATION

Since the results of the response surface plots (FiIGURE 5.1 A) show that the high molasses concentration would inhibit biomass and product formation, fed-batch fermentation was attempted. The aim of the fed-batch process was to avoid the substrate inhibition and enhance the production of lipids by feeding additional substrate. The culture with an initial working volume of 3 L was first operated at batch mode. The initial molasses concentration and C/N ratio were 6 % and 29.80, respectively. After 1 L of 3% molasses was added every 24 h up to 48 hours of fermentation time to maintain molasses concentration at an optimum level of 6%, the fermentation process hard 5 days. The culture pH was not controlled throughout the fermentation process. The agitation speed and aeration rate were controlled at 250 rpm and 1.5 vvm, respectively. The profiles of cell growth, lipid yield and molasses consumption, are shown in Fig. 5.5 and 5.6. The feeding of the substrate increased biomass, lipid content and lipid productivity up to 27.95 g/L, 60.94 % and 0.14 g/L/h, respectively. The molasses consumption was also improved in the fed-batch fermentation because the additional substrate, the cells could utilize more molasses.

FIGURE 5.5 - THE TIME COURSE OF CELL GROWTH AND LIPID PRODUCTION IN A 10 L FERMENTOR BY *R. glutinis* WITH SUGAR CANE MOLASSES BY FED BATCH.

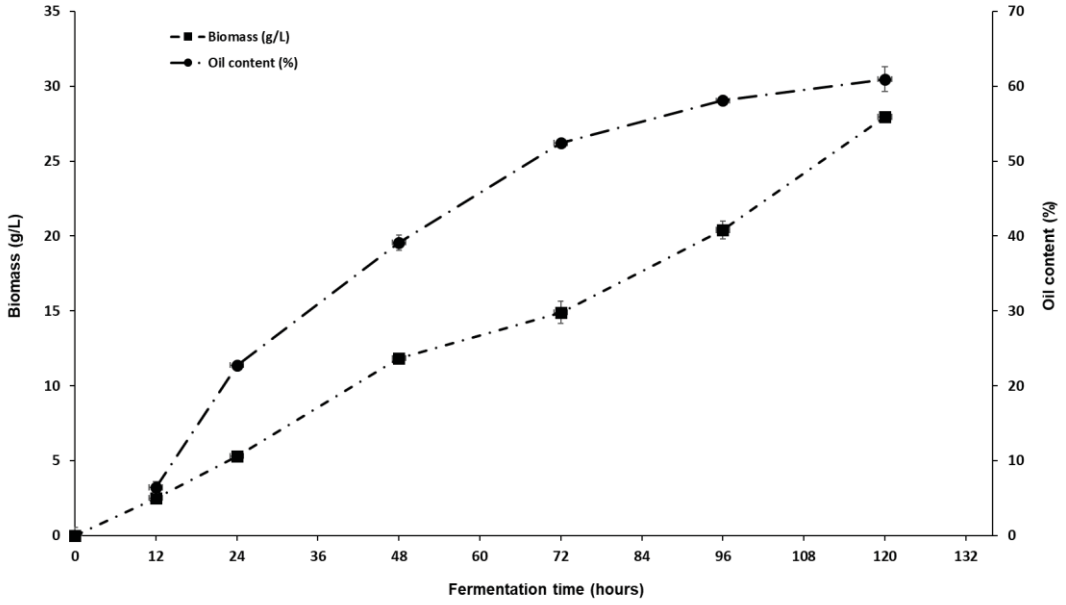


FIGURE 5.6 - THE TIME COURSE SUGARS AND NITROGEN CONSUMPTION, PH NOT CONTROLLED IN A 10 L FERMENTOR WITH R. GLUTINIS WITH SUGAR CANE MOLASSES BY FED BATCH

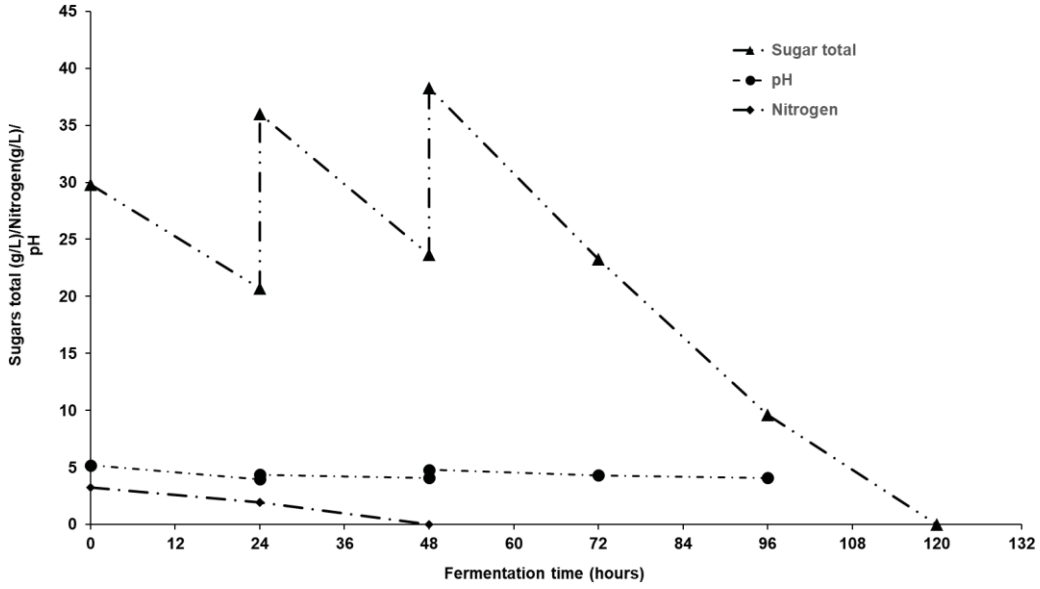


TABLE 5.7, shows the comparison of the performance in flask, batch and fed-batch cultures. The final amount of lipids in the fed-batch was 13.88 g which was about twice of the batch culture (5.31 g). This thus increased the productivity of lipids from 0.042 g/L/h in the batch culture to 0.096 g/L/h in the fed-batch culture. The yields of

lipids on sugar cane molasses decreased in the fed-batch culture compared with the batch culture. This indicated that the cells consumed more molasses to maintain their activities in the fed-batch culture. There are two works made with sugar cane molasses using *Rhodotorula glutinis*, the most recent one was by Wang and Ren (Wang and Ren, 2014) they obtained a productivity of 0.094 g / L/h while in this work we obtained 0.141 g/L/h with *Rhodotorula glutinis* PPGEBB115

TABLE 5.7. LIPID PRODUCTION UNDER DIFFERENT CONDITIONS BY *R. glutinis*

Cultivation method	**Biomass (g/L)**	**Lipid content (%)**	**Lipid titer (g/L)**	**Lipid yield (%)**	**Lipid productivity (g/L.h)**
Optimization batch cuture by shake flask	5.41	27.92	1.51	18.54	0.010
Batch cuture by fermentador (200 rpm)	14.58	31	4.51	15.03	0.045
Batch cuture by fermentador with tween (250 rpm)	9.58	53	5.31	16.31	0.042
Constant nitrogen fed-batch 2 times a feed per day	27.95	60.94	17.01	24.28	0.141

Several authors have concluded that using a batch fermentation process increases the biomass and lipid yield. For example, in the work of Liu et al., (2015) which used *Rhodotorula glutinis* with concorb hydrolysate, they succeeded in increasing the lipid content 5 time more than in a batch fermentation, that is to say, from 5.5 g/L to 30.6 g/L, respectively. Similarly, in the work of Saenge et al., (2011) produced 4.33 g/L in a Batch and 8.36 g/L in a Fed Batch using as substrate glycerol and Wang and Ren (2014) of 6.31 g/L at 7.93 g/L using as substrate sugar cane molasses, all these authors worked with *Rhodotorula glutinis*.

5.4.5. LIPID COMPOSITION

By gas chromatography the composition of the fatty acids of *Rhodotorula glutinis* PPGEBB115 oil was determined from sugar cane molasses. In table 5.8 it can be observed that there is a greater presence of oleic acid (63.59%) and palmitic acid (20.81%). In less quantity is the stearic acid (6.68%) and linoleic acid (5.42%). The amount of different fatty acids exist in the feedstock are critical to the physical properties of biodiesel because the transesterification does not change the fatty acid

composition (Ramos et al., 2009). As Table 5.7 indicated, the main composition of the lipid produced with sugar cane molasses was palmitic acid, stearic acid, oleic acid and linoleic acid. Oleic acid was the most abundant and heptacosanoic acid was the least fatty acid in the case of molasses as substrate. High content of unsaturated fatty acids will lead to satisfactory fuel properties at low temperatures (Zheng et al., 2012). However, excess unsaturated fatty acids play an adverse effect on the oxidative stability of biodiesel (Luque, 2010; Ramírez-Verduzco et al., 2012). The total unsaturated fatty acids of the lipid produced with molasses cane were 70.25 %, that is, there is presence of omega 6 and 9. Which indicated that lipid from oleaginous yeast R. glutinis has potential as a biodiesel feedstock.

TABLE 5.8. LIPID COMPOSITION OF R. GLUTINIS WITH DETOXIFIED HYDROLYSATE

Lipid composition	Value (%)
Lauric acid (C13:0)	0.43
Miristic acid (C14:0)	0.70
Palmitic acid (C16:0)	20.81
Estearic acid (C18:0)	6.68
Oleic acid (C18:1)	63.59
Linoleic acid (C18:2)	5.42
Eicosadienoic acid (C20:2)	0.49
Erucic acid (C22:1)	0.75
Hexacosanoic acid (C26:0)	0.77
Heptacosanoic acid (C27:0)	0.36

5.4.6 POTENTIAL OF *Rhodotorula glutinis* PPGEBB115 OIL FOR THE PRODUCTION OF BIODIESEL.

The amount of fatty acids present in lipids significantly affects the quality of biodiesel, that is, there is an effect on the physical properties of biodiesel because transesterification does not change the composition of fatty acids. For this reason we have obtained several mathematical models described by Hoekman et al. (2012) from unsaturated fatty acid data generated in various research works in oleaginous microorganisms and oleaginous seeds, as can be seen in Table 5.9. *Rhodorotula glutinis* oil complies with US biodiesel standard ASTM D6751 and EU biodiesel standard EN 14214 with regard to viscosity, specific gravity, cetane number and iodine number. The other properties of biodiesel that establish standards will depend on the conditions of the transesterification process, for example, temperature, time, type and amount of catalyst, agitation, among others.

We can also see that the oil properties of yeasts are similar with oil palm and *Jatropha curcas*, which are currently being studied for the production of biodiesel. The advantage of using an oleaginous yeast is that it decreases the time of obtaining of oil compared to a perennial plant that would take between 4 and 5 years, to obtain its maximum production in addition to that it is required great extensions of earth to obtain a greater quantity of seeds.

TABLE 5.9 COMPARISONS OF BIODIESEL PROPERTIES OF OLEAGINOUS YEASTS, OIL PALM, *Jatropha curcas*, WITH RESPECT THE US BIODIESEL AND EU BIODIESEL STANDARDS

Properties	**US biodiesel standard ASTM D6751**	**EU biodiesel standard EN 14214**	***Rhodotorula glutinis* PPGEBB115[a]**	***Jatropha curcas*[b]**	**Oil palm[b]**	***Rhodosporidium toruloides DEBB 5533*[c]**	***Rhodotorula sp.*[d]**
Viscosity (mm/s^2)	1.9 - 6.0	3.5 - 5.0	4.762	4.75	4.61	5.24	4.53
Specific gravity ()	-	0.86 - 0.9	0.8764	0.876	0.873	-	0.8785
Cloud point (°C)	-	-	10.611	5	14	6	5.73
Cetane number	47 min	51 min	58.192	55.7	50.5	-	55.75
Iodine number (max)		120	64.95	109.5	54	61.7	92.13
HHV (MJ/kg)	-	-	39.77	40.7	40.6	-	40.41

Fonte: a. Autor, b. S.K. Hoekman et al. (2012), c. C.R. Soccol et al (2017), d. A. Tanimura et al. (2014)

5.5 CONCLUSIONS

The sugar cane molasses is a by-product obtained from the sugar industry, its price is low and readily available in Brazil. In this research work proved to be a good source of carbon, highly viable for the production of microbial oil from *Rhodotorula glutinis.* In this study, based on the experiments carried out, a concentration of 6% of sugar cane molasses was added, adding 3 g/L of nitrogen source and 1.5 g/L of biosurfactant (Tween 20), obtaining a biomass production of 29.7 g / L, 47% lipid content and a productivity of 0.088 g/L/h, this production was obtained in a fed-batch fermentation process. The microbial oil from *Rhodotorula glutinis* is mainly composed of oleic acid, stearic acid, palmitic acid, linoleic acid mainly, which indicates that it is a viable oil for the production of biodiesel.

5.6 SUGGESTIONS

It is suggested to carry out experiments of the other yeasts that were isolated and identified because they have good cell growth and oil production using sugar cane molasses.

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